UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Interactions between high and low-virulence isolates of *Neospora Caninum* and the bovine placenta

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Laura Jiménez Pelayo

Supervisors

Luis Miguel Ortega Mora
Esther Collantes Fernández
Javier Regidor Cerrillo

Madrid

COMPLUTENSE UNIVERSITY OF MADRID

VETERINARY FACULTY

Animal Health Department

INTERACTIONS BETWEEN HIGH- AND LOW-VIRULENCE ISOLATES OF *NEOSPORA CANINUM* AND THE BOVINE PLACENTA

Supervisors:

Luis Miguel Ortega Mora

Esther Collantes Fernández

Javier Regidor Cerrillo

DOCTORAL THESIS

Ms. Laura Jiménez Pelayo

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UNIVERSIDAD COMPLUTENSE DE MADRID

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INTERACCIONES ENTRE AISLADOS DE ALTA Y BAJA VIRULENCIA DE *NEOSPORA CANINUM* Y LA PLACENTA BOVINA

Directores:

Luis Miguel Ortega Mora

Esther Collantes Fernández

Javier Regidor Cerrillo

TESIS DOCTORAL

Dña. Laura Jiménez Pelayo

Madrid, julio de 2019

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Y dirigida por: D. Luis Miguel Ortega Mora, Dña. Esther Collantes Fernández y D. Javier Regidor Cerrillo

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Madrid, 4 de julio de 2019

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D. Luis Miguel Ortega Mora, Doctor en Veterinaria y Catedrático de Universidad adscrito al Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, Dña. Esther Collantes Fernández, Doctora en Veterinaria y Profesora titular en el Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, y D. Javier Regidor Cerrillo, Doctor en Farmacia e investigador contratado por la empresa Saluvet-innova, con base en el Departamento de Sanidad Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid

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Que la tesis doctoral titulada "Interacciones entre aislados de alta y baja virulencia de *Neospora caninum* y la placenta bovina" que presenta la Licenciada en Veterinaria Dña. Laura Jiménez Pelayo ha sido realizada en las dependencias del Departamento de Sanidad Animal de la Facultad de Veterinaria, de la Universidad Complutense de Madrid bajo su supervisión y cumple todas las condiciones exigidas para optar al grado de Doctor por la Universidad Complutense de Madrid con Mención Internacional.

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1) Realización de una estancia mínima de tres meses en una institución de enseñanza superior o centro de investigación fuera de España:

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- Investigador principal: Prof. Christiane Pfarrer.
- Duración de la estancia: 3 meses (15/09/2017-14/12/2017).

2) Los apartados de resumen, introducción, resultados, discusión y conclusiones de la tesis doctoral han sido redactados en una en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España (inglés).

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"…sueña con metas altas y volarás como las águilas. Eso debes hacer; alcanzar las cumbres de la vida. Busca al que sea sabio y aprende con él. Usa bien la ambición sin por ello dañar a nadie. No hagas que tengan que recriminarte en tu trabajo, hazlo siempre bien. E intenta ganar cuando te hagan competir. No te dejes avasallar por nadie y aunque hayas nacido en un hogar humilde, no te consideres por ello indigno. Si luchas con esfuerzo, conseguirás todo lo que te propongas."

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LIST OF ABBREVIATIONS

ASF	Average size focus	Tamaño medio del foco
BCEC-1	Bovine caruncular epithelial cell line 1	Células epiteliales de la carúncula bovina
BKI	Bumped kinase inhibitor	Inhibidores de la proteína quinasa
BNC/TGC	Binucleated trophoblast cells/Giant trophoblast cells	Células del trofoblasto binucleares/Células del trofoblasto gigantes
bp	Base pairs	Pares de bases
bPL	Bovine placental lactogen	Lactógeno placentario bovino
BUVECs	Bovine umbilical vein endothelial cells	Células endoteliales umbilicales bovinas
BVD	Bovine viral diarrhea	Diarrea vírica bovina
CA	Caruncle	Carúncula
Ca	Calcium	Calcio
CCL	Chemokine (C-C motif) ligand	Ligando de quimioquina (motivo C-C)
CD	Cluster of differentiation	Cúmulo de diferenciación
CDPK	Calcium dependent protein kinase	Proteína quinasa dependiente de calcio
cInfR	Cell infection rate	Tasa de infección celular
cm	Centimetre	Centímetro
CNS/SNC	Central nervous system	Sistema nervioso central
CO	Cotyledon	Cotiledón
CO_2	Carbon dioxide	Dióxido de carbono
CSF	Colony stimulating factor	Factor estimulador de colonias
Ct	Cycle threshold value	Valor umbral de ciclo
DAB	3,3'Diaminobenzidine	3,3'Diaminobenzidina
DC/CD	Dendritic cells	Células dendríticas
DMEM	Dulbecco's modified eagle medium	Dulbecco's modified eagle medium
DNA/ADN	Deoxyribonucleic acid	Ácido desoxirribonucleico
dpi	Days post-infection	Días post-infección
dsDNA	Double-stranded DNA	ADN de doble cadena
e.g.	*Exempli gratia* (for instance)	*Exempli gratia* (por ejemplo)
ECM/MEC	Extracellular matrix	Matriz extracelular
ELISA	Enzyme-linked immunosorbent assay	Ensayo inmunoenzimático
EnTT	Endogenous transplacental transmission	Transmisión transplacentaria endógena
ESTs	Expressed sequence tag	Etiqueta de secuencia expresada
EU/UE	European Union	Unión Europea
ExTT	Exogenous transplacental transmission	Transmisión transplacentaria exógena
F3	Bovine placental trophoblast cell line	Células del trofoblasto bovino
FB	Foetal brain	Cerebro fetal
FCS	Foetal calf serum	Suero fetal bovino
FGF	Fibroblast growth factor	Factor de crecimiento de fibroblastos
FL	Foetal liver	Hígado fetal
GRA	Dense granule antigen	Antígeno de gránulos densos
h	Hours	Horas
HE	Haematoxylin-eosin	Hematoxilina-eosina
HFF	Human foreskin fibroblasts	Fibroblastos de piel de prepucio humana
HPCVE	Human placental chorionic villi explants	Explantes de vellosidades coriónicas placentarias humanas
hpi	Hours post-infection	Horas post-infección
i.e.	*Id est* (this is)	*Id est* (esto es)
ICAM	Intercellular adhesion molecule	Molécula de adhesión intercelular

IFAT	Indirect fluorescent antibody test	Test de inmunofluorescencia indirecta
IFN	Interferon	Interferón
IgG	Immunoglobulin G	Inmunoglobulina G
IHC/IHQ	Immunohistochemistry	Inmunohistoquímica
IL	Interleukin	Interleuquina
IM	Intramuscular	Intramuscular
iNOS	Inducible nitric oxide synthase	Óxido nítrico sintasa inducible
IP	Intraperitoneal	Intraperitoneal
ITS-1	Internal transcribed spacer-1	Espacio transcrito interno-1
IV	Intravenous	Intravenoso/a
K	Potassium	Potasio
LAK	Lymphokine activated killer cell	Célula linfoide derivada de las células NK
Lymphocyte Th	T helper lymphocyte	Linfocito T colaborador
M	Molar	Molar
MAPK	Mitogen-activated protein kinase	Proteína quinasa activada por mitógenos
M-CSF	Macrophage colony-stimulating factor	Factor estimulante de colonias de macrófagos
MEK/ERK	Mitogen-activated protein kinase/Extracellular signal-regulated kinase	Proteína quinasa activada por mitógenos / Quinasa regulada por señal extracelular
MHC	Major histocompatibility complex	Complejo mayor de histocompatibilidad
MIC	Microneme protein	Proteína de micronemas
min	Minute	Minuto
ml	Millilitre	Mililitro
mM	Milli molar	Milimolar
mm	Millimetre	Milímetro
MMP	Matrix metalloproteinase	Metaloproteinasas de la matriz extracelular
MOI	Multiplicity of infection	Multiplicidad de infección
MPSS	Massively parallel signature sequencing	Secuenciación masiva de firmas paralelas
mRNA/ARNm	Messenger ribonucleic acid	Ácido ribonucleico mensajero
n	Sample number	Número de muestra
NA	Not applicable	No aplicable
Na	Sodium	Sodio
Nc	*Neospora caninum* (prefix)	*Neospora caninum* (prefijo)
NC	Data not comparable	Datos no comparables
NF	Necrotic foci	Focos de necrosis
NGS	Normal goat serum	Suero normal de cabra
NK	Natural killer (cells)	(Células) asesinas naturales
NO	Nitric oxide	Óxido nítrico
No.	Number	Número
NOD	Nucleotide-binding oligomerization domain-like receptors	Receptores similares al dominio de oligomerización de unión a nucleótidos
NS	No significant differences	Diferencias no significativas
NVF	Non-viable foetuses	Fetos no viables
O_2	Oxigen	Oxígeno
OD	Optical density	Densidad óptica
OR	Odds ratio	Odds ratio
PAF	Platelet-activating factor	Factor activador de plaquetas
PAGs	Pregnancy-associated glycoproteins	Glicoproteínas asociadas a la gestación

PBS	Phosphate buffer saline	Tampón fosfato salino
PCR	Polymerase chain reaction	Reacción en cadena de la polimerasa
pg	Picogram (10-12 grams)	Picogramo (10-12 gramos)
pi	Post-infection	Post-infección
pInvR	Parasite invasion rate	Tasa de invasión del parásito
pInvR$_T$	Total parasite invasion rate	Tasa de invasión total del parásito
PRP	Prolactin related protein	Proteína relacionada con la prolactina
PRRs	Pattern recognition receptors	Receptores de reconocimiento de patrones
qPCR	Quantitative PCR	PCR cuantitativa
RAPD	Randomly amplified polymorphic DNA	ADN polimórfico amplificado al azar
RIPC	Relative index per cent	Índice relativo por cien
RNA/ARN	Ribonucleic acid	Ácido ribonucleico
ROP	Rhoptry bulb protein	Proteína de cuerpo de roptrias
ROS	Reactive oxygen species	Especies de oxígeno reactivas
RT	Room temperature	Temperatura ambiente
SAG	Surface antigen	Antígeno de superficie
SAGE	Serial analysis gene expression	Análisis serial de expresión génica
SC	Subcutaneous	Subcutánea
SD	Standard deviation	Desviación estándar
SERP	Serpine	Serpina
SRS	SAG1-related sequence	Secuencia SAG1 relacionada
Td	Doubling time	Tiempo de duplicación
Tg	*Toxoplasma gondii* (prefix)	*Toxoplasma gondii* (prefijo)
TGF	Transforming growth factor	Factor transformante de crecimiento
TIMP	Matrix metalloproteinase inhibitor	Inhibidores de las metaloproteinasas de matriz extracelular
TLR	Toll-like receptor	Receptor tipo toll
TNF	Tumoral necrosis factor	Factor de necrosis tumoral
TY	Tachyzoite yield	Recolección de taquizoítos
UK	United Kingdom	Reino Unido
USA	United States of America	Estados Unidos de América
UTC	Uninucleated trophoblast cells	Células del trofoblasto mononucleares
VCAM	Vascular cell adhesion molecule	Molécula de adhesión vascular
VEGF	Vascular endothelial growth factor	Factor de crecimiento endotelial
VF	Viable foetuses	Fetos viables
WB	Western blotting	Western blot
wpi	Weeks post-infection	Semanas post-infección
µg	Micrograms (10^{-6} grams)	Microgramo (10^{-6} gramos)
µm	Micrometer	Micrómetros
X^2	Chi square	Chi cuadrado
%LES	Total area affected by necrosis	Área total afectada por la necrosis
°C	Grades centigrade	Grados centígrados
2-DE	Two-dimensional electrophoresis	Electroforesis en dos dimensiones

CHAPTER I

SUMMARY

RESUMEN

Bovine neosporosis is one of the most important causes of abortion in cattle worldwide and *Neospora caninum*, an apicomplexan protozoan parasite, is the causal agent of the disease. Millionaire economic losses have been estimated in infected cattle, mainly associated with the reproductive failure. *N. caninum* is very effective at crossing the placental barrier and placental damage is crucial in the pathogenesis of abortion. In addition, infection during pregnancy poses a challenge for the immune system of pregnant cows. Changes in the T helper 1 and T helper 2 balance in the placenta during gestation have been associated with abortion. Bovine trophoblast and caruncular cell layers are key cellular components at the maternal-foetal interface in placentomes, playing a fundamental role in placental functionality. In addition to their role as a barrier, those cells are able to recognize and induce immune responses against *N. caninum,* participating in the initiation of innate immune responses at the placental level as well as in the development of an adaptative immune response. However, the causes that lead to abortion remain unknown and the mechanisms by which *N. caninum* infects the placenta and reaches the foetus are poorly studied. Despite bovine placenta plays a fundamental role in the pathogenesis of bovine neosporosis and placental invasion, proliferation and dissemination to the foetus seem to be crucial events, few studies focused on the early host-cell interactions at the placental level have been approached. In fact, only one descriptive *in vitro* study in primary giant trophoblast cells was carried out up to date. Moreover, a limited number of *in vivo* studies have been conducted to investigate the consequences of *N. caninum* infection at mid-gestation, which is when most abortions occur in naturally infected cattle. Finally, the parasite intra-specific variability influences the outcome of infection. However, studies conducted to compare isolates of different virulence are scarce, and none has assessed comparisons of isolates in bovine placental target cells at early stages of infection neither *in vitro* or *in vivo.* Here, the role of the placenta in the pathogenesis of the bovine neosporosis and the influence of the biological variability of the isolate in placental damage have been investigated.

The first objective of the present Doctoral Thesis was the characterization of the interactions between high- and low-virulence isolates of *N. caninum* and bovine placental target cells *in vitro* with the aim of clarifying the events happened in the placenta and the role played by cells conforming the maternal-fetal interface after infection with isolates of different virulence of *N. caninum,* as well as the factors that enable some isolates to be more effectively transmitted and cause fetal death than others. In order to reach this objective, we initially studied tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence *N. caninum* isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells by immunostaining plaque assay and real-time PCR (qPCR). *N. caninum* invaded and proliferated in both cell lines. However, invasion, infection and proliferation of both isolates were lower in BCEC-1 than in F3, suggesting resistance of caruncular layer to *N. caninum* infection and a role as a barrier for this cell line. Tachyzoites of both isolates showed an early egression in BCEC-1, showing that BCEC-1 cannot avoid transmission although they can restrict adhesion and multiplication. On the other hand, Nc-Spain7 showed higher invasion and infection than Nc-Spain1H, mainly in trophoblast cells, as well as higher proliferation abilities, as was demonstrated in previous stablished cell cultures. Parasitophorous vacuoles raised a considerably big size in bovine trophoblast, contrary to observed in BCEC-1, that may indicate an important role of foetal trophoblasts as a niche for *N. caninum* replication.

After studying the events of the lytic cycle, modulation of the trophoblast and caruncular immune responses by both parasites was investigated *in vitro* by RNA extraction and RT-qPCR determination in order to establish the role of placental cells in the regulation of innate immune responses at the placental level and their reprogramming by *N. caninum* infection with isolates of variable virulence. Expression profiles of toll-like receptor-2 (TLR-2), Th1 and Th2 cytokines (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17, IFN-γ, TGF-β1, TNF-α), and endothelial adhesion molecules (ICAM-1 and VCAM-1) were investigated. A similar modulation in both cell lines was observed consisting in upregulation of pro-inflammatory TNF-α and IL-8 and downregulation of IL-6 and TGF-β1 in infected cultures. Protein secretion of IL-6, IL-8 and TNF-α was confirmed by ELISA. Therefore, *N. caninum* infection favoured a pro-inflammatory response in placental target cells *in vitro*. Comparing isolates, higher expression levels of TLR-2 and TNF-α were found in trophoblasts infected with the low-virulence isolate Nc-Spain1H, suggesting immunomodulation differences between high- and low-virulence isolates, which would partially explain the existing differences in virulence.

The study of the early infection in a pregnant bovine model inoculated with high- and low-virulence isolates of *N. caninum* at mid-gestation was the second objective of this Doctoral Thesis. Host-pathogen interaction, specially in the first stages of infection, as well as the intrinsic virulence factors associated with the implicated isolate are crucial in the outcome of infection. To achieve this objective, an experimental infection was carried out by intravenously inoculation of 10^7 tachyzoites of Nc-Spain7 or Nc-Spain1H isolates in pregnant heifers, and subsequent culling of the animals at two early moments post-infection (10 and 20 days post-infection-dpi). Comparative analysis of the early infection dynamics (clinical outcome, parasite distribution and burden and lesion development in placental and foetal tissues), the specific antibody response and the early modulation of placental immune responses and the extracellular matrix (ECM) regulation were investigated. This study brings light into the role played by the immune responses at the maternal-fetal inteface in the clinical outcome or in the control of parasite transmission and proliferation in placental and foetal tissues and provide new insight into the host-pathogen interactions, validating at the same time the results obtained *in vitro*.

Differences in the outcome of the infection with high- and low-virulence isolates were found. Fever was the first clinical sign associated with the infection. Nc-Spain7-infected animals presented a biphasic increase of the body temperature, with two peaks at 1 and 3 dpi, and 100% of animals were febrile. Differences in the febrile responses induced by high- and low-virulence isolates may be explained by a more efficient multiplication of Nc-Spain7 as was previously indicated and confirmed in placental cultures *in vitro*. Early seroconversion and higher levels of IFN-γ in sera, detected by ELISA, were also related to the high virulent isolate. However, these levels did not correlate with protection against the parasite since earlier and wider dissemination of Nc-Spain7 (10 dpi) and higher parasite burden (detected by PCR and qPCR respectively), and more severe lesions in placental and foetal samples (observed by microscopic histological examination), were found in Nc-Spain7-infected animals than in Nc-Spain1H. In addition, 40% of foetal death was detected in the heifers infected with Nc-Spain7 when they were culled at 20 dpi. Remarkably, the modulation of the local immune responses and the ECM of the placenta were also influenced by the parasite isolate. Elements of the innate and adaptative immune responses as pattern recognition receptors (PRRs), cytokines, chemoattractant genes and ECM components were studied in placental samples by immunohistochemistry (IHC) and qPCR. Nc-Spain7 was not detected by the placental PRRs traditionally implicated in intracellular parasite recognition at 10 dpi. Subsequently, this isolate did not induce upregulation of any investigated gene except for

IFN-γ, IL-4 and TNF-α. However, parasite replication and lesion development at 20 dpi probably led to PRRs activation and exacerbated expression of cytokines (mainly IFN-γ, iNOS, TNF-α and IL-12p40) and chemokines, with a Th1/Th2 balance displaced towards a predominant Th1 response, that probably contributes to foetal death. Moreover, IL-8, TNF-α and iNOS were upregulated and TGF-β1 downregulated in placental tissues from non-viable foetuses, suggesting that they may be directly related to *N. caninum* abortion. Expression of cytokines TGF-β1, IL-6 and IL-17A in bovine placenta was not upregulated by Nc-Spain7 infection at any time, indicating the lack of activation of certain repair processes. In addition, a profound alteration of the ECM, with decreased expression of MMP-2 and TIMP-2 and destruction of collagen, fibronectin and vimentin could contribute to the abortion mechanism. On the other hand, the low virulent isolate Nc-Spain1H induced upregulation of PRRs, cytokines, chemoattractant genes and ECM modulators as early as 10 dpi. This modulation was maintained at 20 dpi at constant levels, maintaining Th1/Th2 responses balanced. Rapid immune responses together with a minor replication ability of Nc-Spain1H may be the causes of the low burden found. In addition, a profound modulation of the ECM by Nc-Spain1H has been evidenced in the placenta *in vivo*. Altogether, different mechanisms seem to be implicated in high- and low-virulence isolates transmission. We propose that Nc-Spain7 arrives to the foetus using its efficient replication ability of and an evasion strategy of the placental immune response in the early stage of the infection. However, high multiplication leads to placental damage and exacerbated immune responses that may be the causes of the abortion. On the other hand, the low-virulence isolate is quickly recognized and controlled by the placental immune responses, which along with the lower proliferation of this isolate, mainly in caruncular cells, suggest a different mechanism to cross the placental barrier. Modulation of the ECM and the hijacking of the immune cells may be the strategies used by this parasite in order to be transmitted to the offspring avoiding placental damage.

La neosporosis bovina es una de las causas más importantes de aborto en el ganado vacuno a nivel mundial. Dicha enfermedad está causada por el protozoo apicomplejo *Neospora caninum*, un parásito intracelular obligado formador de quistes tisulares, estrechamente relacionado con *Toxoplasma gondii*. La infección con este parásito causa cuantiosas pérdidas económicas en todo el mundo, principalmente asociadas al fallo reproductivo. *N. caninum* es capaz de cruzar la barrera placentaria de forma muy eficaz y el daño producido en la placenta al transmitirse al feto es crucial en la patogénesis del aborto. Además, la infección durante la gestación representa un desafío para el sistema inmunológico de los animales infectados. De hecho, desequilibrios en el balance de la respuesta de tipo Th1 y Th2 en la placenta durante la gestación se han asociado con el aborto. Las células del trofoblasto bovino junto con las células epiteliales de la carúncula componen la interfaz materno-fetal de los placentomas, desempeñando un papel fundamental en la funcionalidad de la placenta. Además de su papel como barrera física, impidiendo el paso de sustancias tóxicas y agentes patógenos de la madre al feto, estas células son capaces de reconocer e inducir respuestas inmunitarias frente a *N. caninum*, participando en la iniciación de respuestas inmunitarias innatas a nivel de la placenta, así como en el desarrollo de una respuesta inmunitaria adaptativa. Sin embargo, las causas que conducen al aborto y los mecanismos por los cuales *N. caninum* infecta la placenta y se transmite al feto siguen siendo desconocidos. A pesar de que la placenta bovina desempeña un papel fundamental en la patogénesis de la neosporosis y de que la invasión de la placenta, junto con la proliferación del parásito en ésta, y su diseminación al feto parecen ser eventos cruciales en el desarrollo de esta enfermedad, hasta el día de hoy se han abordado pocos estudios centrados en las interacciones tempranas entre las células hospedadoras y la placenta. De hecho, se dispone de un único estudio *in vitro,* descriptivo y muy limitado, en células multinucleadas de trofoblasto bovino. Además, el número de investigaciones *in vivo* explorando las consecuencias de la infección por *N. caninum* a mitad de la gestación, cuando se producen la mayoría de los abortos en vacas infectadas naturalmente, es muy limitado. Finalmente, la variabilidad intraespecífica del parásito influye en el resultado de la infección. Sin embargo, los estudios realizados comparando aislados de distinta virulencia son escasos, y ningún estudio abordado anteriormente había comparado aislados de distinta virulencia en células diana de la placenta bovina en las primeras etapas de la infección. Teniendo en cuenta lo expuesto previamente, en la presente Tesis Doctoral se ha investigado el papel de la placenta en la patogenia de la neosporosis bovina y la influencia de la variabilidad biológica del aislado en el daño placentario.

El primer objetivo de la presente Tesis Doctoral consistió en la caracterización de la interacción parásito-hospedador entre aislados de alta y baja virulencia de *N. caninum* y células diana de la placenta bovina *in vitro.* El fin de dicho objetivo fue identificar los eventos ocurridos en la placenta y el papel desempeñado por las células que conforman la interfaz materno-fetal después de la infección con aislados de distinta virulencia, así como elucidar los mecanismos utilizados por el parásito para atravesar la placenta y los factores que permiten que algunos aislados se transmitan de manera más efectiva que otros, causando la muerte fetal. Para alcanzarlo, en primer lugar, se estudiaron los mecanismos de adhesión, invasión, proliferación y egresión de los taquizoítos de los aislados Nc-Spain7 (de alta virulencia) y Nc-Spain1H (de baja virulencia) en cultivos establecidos de células epiteliales carunculares bovinas (BCEC-1) y trofoblásticas (F3) mediante ensayos de inmunofluorescencia en placa y de PCR en tiempo real (qPCR). *N. caninum* invadió y proliferó en ambas líneas celulares. Sin embargo, la invasión, la infección y la proliferación de

ambos aislados fueron menores en BCEC-1 que en F3, lo que sugiere que las células carunculares maternas son resistentes a la infección por *N. caninum* y dejan entrever el papel de barrera que tendría esta línea celular en la interfaz materno-fetal. Además, los taquizoítos de ambos aislados mostraron mecanismo de egresión temprana en BCEC-1, lo que demuestra que a pesar de que estas células pueden restringir la adhesión y la multiplicación del parásito, no pueden evitar su transmisión. Por otro lado, el aislado más virulento, Nc-Spain7, presentó una mayor capacidad de invasión y de infección que el aislado menos virulento, Nc-Spain1H, principalmente en células trofoblásticas, así como una mayor capacidad de proliferación. Las vacuolas parasitóforas alcanzaron un gran tamaño en las células del trofoblasto bovino, contrariamente a lo observado en las células carunculares, lo que podría indicar una importante implicación de los trofoblastos en la infección por *N. caninum*, siendo probablemente el nicho de replicación del parásito.

Después de estudiar los eventos del ciclo lítico, se investigó la modulación de las respuestas inmunitarias del trofoblasto y la carúncula *in vitro,* mediante la extracción de ARN y RT-qPCR, con el objetivo de establecer el papel de las células de la placenta en la regulación de la respuesta inmunitaria innata y su reprogramación por la infección con aislados de distinta virulencia de *N. caninum*. Se investigaron los perfiles de expresión del receptor *toll-like* 2 (TLR-2), de citoquinas de tipo Th1 y Th2 (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17, IFN-γ, TGF-β1, TNF-α), así como de moléculas de adhesión endotelial (ICAM-1 y VCAM-1), encontrándose una regulación similar en ambas líneas celulares. Dicha modulación consistió en un aumento de expresión del TNF-α y la IL-8 y en un descenso de la IL-6 y del TGF-β1 en los cultivos infectados. La secreción proteica de IL-6, IL-8 y TNF-α fue confirmada por ELISA. Por lo tanto, se determinó que la infección por *N. caninum* favoreció una respuesta proinflamatoria en las células diana de la placenta bovina *in vitro*. Cabe destacar que las comparaciones entre los aislados mostraron una mayor expresión del TLR-2 y del TNF-α en trofoblastos infectados con el aislado de baja virulencia Nc-Spain1H, lo que sugirió mecanismos de modulación distintos con cierta evasión de la respuesta inmunitaria por parte del aislado más virulento. Esto podría suponer una explicación, al menos parcial, a las diferencias biológicas observadas entre los aislados.

El estudio de la infección temprana en un modelo bovino gestante inoculado con aislados de alta y baja virulencia de *N. caninum* en el segundo tercio de la gestación supuso el segundo objetivo de la presente Tesis Doctoral. La interacción parásito-hospedador, especialmente en las primeras etapas de la infección, así como los factores de virulencia intrínsecos asociados con el aislado implicado son cruciales en el resultado de la infección. Para lograr este objetivo se realizó una infección experimental mediante la inoculación intravenosa de 10^7 taquizoítos de los aislados Nc-Spain7 o Nc-Spain1H en novillas gestantes, y el posterior sacrificio de los animales en dos momentos tempranos tras la infección (10 y 20 días después de la infección - dpi). El análisis comparativo de la dinámica temprana de la infección (resultado clínico, distribución y carga parasitaria y desarrollo de lesiones en tejidos placentarios y fetales), de la respuesta humoral específica y de la modulación precoz de las respuestas inmunitarias de la placenta y la regulación de la matriz extracelular (MEC) ponen de manifiesto el papel que desempeñan las respuestas inmunitarias de la interfaz materno-fetal en el resultado clínico o en el control de la transmisión y proliferación del parásito en los tejidos placentarios y fetales, y proporcionan una nueva visión de las interacciones parásito-hospedador, validando al mismo tiempo los resultados obtenidos *in vitro*.

Se observaron diferencias en el resultado de la infección con aislados de alta y baja virulencia de *N. caninum*. El aumento de la temperatura corporal fue el primer signo clínico asociado con la infección. El 100% de los animales infectados con el aislado Nc-Spain7 presentaron fiebre, con una dinámica bifásica consistente en dos picos de fiebre a los 1 y 3 dpi. Las diferencias en las respuestas febriles inducidas por los aislados de alta y baja virulencia pueden explicarse por una multiplicación más eficiente del aislado más virulento, como ya fue indicado previamente y confirmado por los resultados de la presente Tesis Doctoral en cultivos de placenta bovina *in vitro*. La seroconversión temprana y los niveles más altos de IFN-γ en suero, detectados por ELISA, también se relacionaron con el aislado más virulento. Sin embargo, estos niveles no se correlacionaron con la protección frente al parásito, ya que en animales infectados con el aislado Nc-Spain7 se observó una mayor y más temprana diseminación del parásito (10 dpi), así como mayores cargas parasitarias, detectadas por PCR y qPCR respectivamente, además de lesiones más graves en muestras de placenta y fetos, observadas mediante examen histológico, que en los animales infectados con el aislado Nc-Spain1H. También se detectó un 40% de mortalidad fetal en las novillas infectadas con el aislado Nc-Spain7 cuando fueron sacrificadas a los 20 dpi. La modulación de la respuesta inmunitaria y de la MEC de la placenta también fueron influenciadas por el aislado del parásito. Elementos de la respuesta inmunitaria innata y adaptativa, como receptores de reconocimiento de patrones (PRRs), citoquinas, quimioquinas y componentes de la MEC, fueron estudiados en muestras de placenta mediante inmunohistoquímica y qPCR. El aislado Nc-Spain7 no fue detectado a los 10 dpi por los receptores de la placenta tradicionalmente implicados en el reconocimiento de parásitos intracelulares. En consecuencia, este aislado no indujo un aumento de la regulación de ningún gen investigado, salvo el IFN-γ, la IL-4 y el TNF-α. Sin embargo, la replicación del parásito y el desarrollo de lesiones observado a los 20 dpi condujeron probablemente a la activación de los receptores de reconocimiento en la placenta y a un aumento exacerbado de la expresión de citoquinas (principalmente IFN-γ, iNOS, TNF-α e IL-12p40) y quimioquinas. El equilibrio de las respuestas de tipo Th1/Th2 fue desplazado hacia una respuesta predominantemente Th1, que probablemente contribuyó a la muerte fetal. Además, la expresión de IL-8, TNF-α e iNOS se encontró aumentada y la expresión del TGF-β1 disminuida en los placentomas de fetos no viables a los 20 dpi, lo que sugiere una relación directa entre la modificación de estos genes y la presencia de aborto inducido por la infección de *N. caninum*. La infección con el aislado virulento no aumentó la expresión de las citoquinas TGF-β1, IL-6 e IL-17A en la placenta bovina en ningún momento tras la infección, lo que indica la falta de activación de ciertos procesos de reparación tissular. Además, una alteración profunda de la MEC, con disminución de la expresión de MMP-2 y TIMP-2 y destrucción de colágeno, fibronectina y vimentina podría contribuir al mecanismo del aborto. Por otro lado, el aislado de baja virulencia Nc-Spain1H indujo un aumento en la expresión de los receptores de reconocimiento, citoquinas, quimioquinas y moduladores de la MEC en un estadio muy temprano de la infección (10 dpi). Esta modulación se mantuvo a los 20 dpi a niveles más o menos constantes, manteniendo equilibradas las respuestas Th1/Th2 a lo largo de todo el periodo experimental. La activación temprana de la respuesta inmunitaria, junto con la menor capacidad de replicación, pueden ser las causas de la baja carga parasitaria encontrada en los animales infectados con el aislado Nc-Spain1H. Además, el aislado Nc-Spain1H indujo una profunda modulación de la MEC de la placenta *in vivo*. El conjunto de todos los resultados observados sugiere un mecanismo de transmisión diferente en aislados de alta y baja virulencia. El aislado más virulento, Nc-Spain7, podría llegar al feto utilizando su eficiente capacidad de replicación y un mecanismo de evasión de la respuesta

inmunitaria de la placenta en la fase inicial de la infección. Sin embargo, la eficiente multiplicación del parásito provocaría daño tisular en la placenta, lo que podría ser la causa de la respuesta inmunológica exagerada, que, a su vez, podría ser una causa desencadenante del aborto. Por otro lado, el aislado de baja virulencia es rápidamente reconocido y controlado por la respuesta inmunitaria de la placenta, lo que unido a la menor proliferación de este aislado, principalmente en las células carunculares, sugieren un mecanismo diferente para cruzar la barrera placentaria. La modulación de la MEC y el secuestro de células inmunitarias podrían ser las estrategias utilizadas por este parásito para ser transmitido evitando el daño placentario.

CHAPTER II

INTRODUCTION

INTRODUCCIÓN

1. *Neospora caninum* and bovine neosporosis

Bovine neosporosis is a parasite disease that causes reproductive failure in cattle and is considered one of the main transmissible causes of abortion in this species leading to significant economic losses both in the dairy and the meat industry worldwide (Reichel *et al.,* 2013). Since *Neospora caninum*, etiologic agent of bovine neosporosis, was recognised for the first time in dogs with severe nervous disorders in Norway (Bjerkås *et al.,* 1984), this parasite has been deeply studied and neosporosis has emerged as a serious disease of cattle worldwide. The interest by *N. caninum* has increased in the veterinary field along the years, having conducted numerous investigations on the biology of the parasite, as well as on the diagnosis, epidemiology and control of the disease (Dubey *et al.,* 2007; Dubey & Schares, 2011; Ortega-Mora *et al*., in press).

It was not until 1988 when Dubey *et al*. proposed the description of a new genus, *Neospora*, and species, *N. caninum*, after identifying the parasite in dogs that presented neuromuscular clinical signs identical to those observed in 1984 (Dubey *et al.,* 1988a). In 1989, *N. caninum* was described as the etiologic agent of bovine neosporosis after the observation of *N. caninum*-like protozoan organisms in the brain and the kidney of necropsied foetuses coming from aborted Holstein dairy cattle in New Mexico (Thilsted & Dubey, 1989). In subsequent years, *N. caninum* infection was described and associated with reproductive failure in cattle in many countries (Anderson *et al.,* 1991; Barr *et al.,* 1991b; Dubey & Lindsay, 1996). Transplacental transmission in the bovine species was experimentally demonstrated in 1992 by inoculation of *N. caninum* tachyzoites in three cows and detection of the parasite in two aborted foetuses (Dubey *et al.,* 1992). One year later, Conrad *et al*. (1993a) obtained the first isolate of *N. caninum* from an aborted bovine foetus, that was used to experimentally infect pregnant cows and reproduce typical clinical signs of bovine neosporosis (Barr *et al.,* 1994).

The definitive host for *N. caninum* was not discovered until 1998 (McAllister *et al.,* 1998), when the presence of *N. caninum* oocysts in dog faeces was evidenced. This finding was later confirmed by Lindsay *et al*. (1999), completing the biological cycle of the parasite. In 1998, *Neospora hughesi*, a second species of *Neospora*, was isolated from the nervous tissue of an adult horse with nervous clinical signs (Marsh *et al.,* 1996; Marsh *et al.,* 1998), and several authors have indicated structural, antigenic, genetic and pathogenic differences between the two species of the genus *Neospora* (Marsh *et al.,* 1998; Walsh *et al.,* 2001).

1.1 Taxonomic classification and morphology of the parasite

Closely related to the zoonotic apicomplexan parasite *Toxoplasma gondii*, *N. caninum* is an obligate intracellular protozoan belonging to the subphylum Apicomplexa (Adl *et al.,* 2012), which includes more than 6.000 species that parasitize practically all groups of animals. The organisms classified within this subphylum are characterized by the presence of an apical complex. More specifically, *N. caninum* belongs to the Sarcocystidae family because of the formation of tissue cysts in the hosts, the presence of heteroxeneous biological cycles with phases of sexual and asexual replication and the oocysts sporulation in the environment (Tenter *et al.,* 2002). The taxonomic classification of *N. caninum* and its main characteristics are summarized in Figure 1. In addition to *N. caninum*, which mainly affects ungulates (Dubey *et al.,* 1988a), the species *N. hughesi* also belongs to the *Neospora* genus. The exact phylogenetical relationship of *N. caninum*

to other members of the Apicomplexa has been, and still is, under controversy (Dubey *et al.,* 2002; Heydorn & Mehlhorn, 2002).

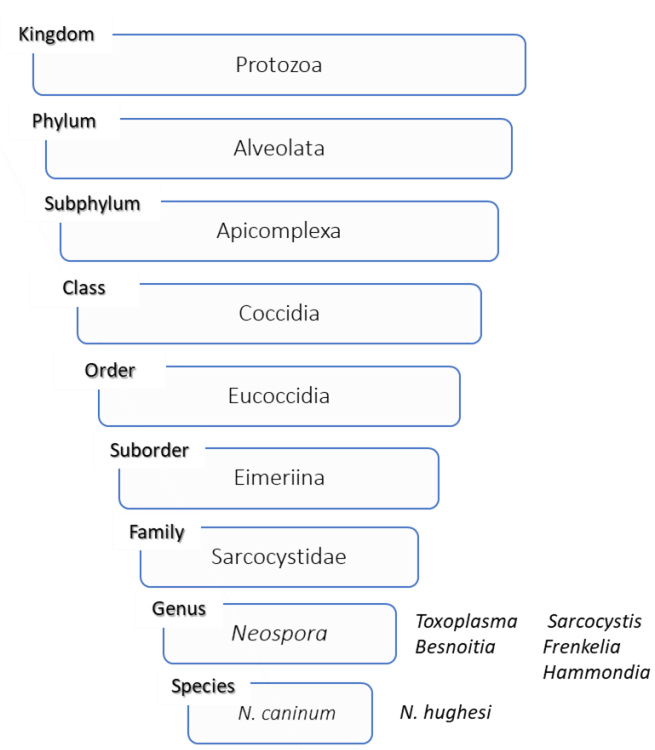


Figure 1. Taxonomic classification of *N. caninum*

Up to date, three invasive stages of the parasite have been described: tachyzoites, bradyzoites within tissue cysts and sporozoites within oocysts (Figure 2). Tachyzoites and tissue cysts containing bradyzoites are the invasive stages located in intermediate host´s tissues whereas unsporulated oocysts are eliminated with definitive host´s faeces, sporulating in the environment and becoming infective.

The tachyzoite is the fast-replicating stage of the parasite being responsible for the acute phase of infection and the damage in the intermediate host (Dubey *et al.,* 2006). Progressive cycles of intracellular replication, tachyzoite release by egression with host cell lysis and infection of surrounding cells, together with related immunopathologic repercussions lead to lesion formation and, in some animals, clinical disease (Dubey *et al.,* 2007). The tachyzoites are able to invade a wide variety of cell types such as neurons, macrophages, dendritic cells (DC), fibroblasts, endothelial cells, myocytes and hepatocytes, allowing a rapid and effective intra-organic dissemination (Barr *et al.,* 1993; Dubey *et al.,* 2002). In addition, the tachyzoite is the most studied and widely used stage of the parasite since its maintenance and growth *in vitro* is relatively simple.

The bradyzoite is the slow-replicating stage of the parasite, responsible for the chronic phase of the infection in the intermediate host. After rapid multiplication of the tachyzoites, they differentiate into bradyzoites, the quiescent life stage of the parasite that forms under host immune pressure and produces a tissue cyst in the intracytoplasmic space of a single cell (Goodswen *et al.,* 2013). Tissue cysts are mainly located in immunopriviledged tissues as central nervous system (CNS) and skeletal muscle tissue (Barr *et al.,* 1991b; Dubey & Lindsay, 1996; Peters *et al.,* 2001; Dubey, 2003), and facilitate long term parasite persistence and chronic

asymptomatic infections (Hemphill *et al.,* 2006; Dubey *et al.,* 2007; Dubey & Schares, 2011). Bradyzoite stage may switch to tachyzoite stage under some conditions such as immunosuppression or immunomodulation, situation that is well documented to occur in pregnant animals and permits tachyzoite spread to other tissues, including dissemination across the placenta and infection of the foetus (Williams *et al.,* 2009).

Finally, sporozoites are contained within the oocyst, which is released to the environment in the faeces of a definitive host and supposes the environmentally resistant form of the parasite. The oocyst sporulates in the environment after approximately 24 hours under favorable conditions of temperature and humidity and constitutes the infective phase for the intermediate host, being responsible for the horizontal transmission between a definitive and an intermediate host. Therefore, oocysts are the key in the epidemiology of neosporosis, but there are only a few reports of *N. caninum* oocyst shedding by naturally infected dogs (reviewed by Dubey *et al*., 2017).

The sporulated oocyst is composed by two sporocysts with four sporozoites each and may contain a residual sporocystic body (McAllister *et al.,* 1998; Lindsay *et al.,* 1999; Dubey *et al.,* 2002). Although the presence of entero-epithelial stages of the parasite in naturally infected dogs has been demonstrated (Kul *et al.,* 2015), the stages of merozoite and gametocyte, widely studied in *T. gondii*, have not been well characterized in *N. caninum* (Dubey *et al.,* 1998; Hehl *et al.,* 2015).

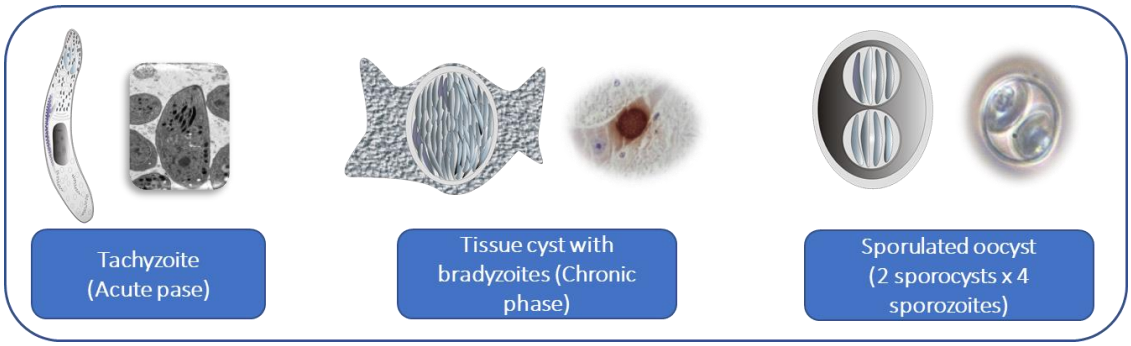


Figure 2. Graphical representation and microscopic images of *N. caninum* parasite stages

Ultrastructurally, all three zoites present, in addition to the typical organelles of any eukaryotic cell, all the characteristic structures of the apicomplexan parasites (Figure 3). The outermost layer of the zoites is the membrane, composed by an external layer that constitutes the cytoplasmatic membrane, and two internal ones which interact with the glideosome, structure involved in the invasion of the host cell (Boucher & Bosch, 2015), and the subpellicular microtubes (Ouologuem & Roos, 2014). The subpellicular microtubules are anchored to the polar rings, acting as a cytoskeleton and allowing the movement of the zoite together with the conoid (Speer & Dubey, 1989; Lindsay *et al.,* 1993; Speer *et al.,* 1999). In the cytoplasm of apicomplexan parasites rhoptries, micronemes, dense granules and amylopectin granules (polysaccharide reserve) are found and their number and distribution confer taxonomic value. The rhoptries have a main function in the formation and maturation of the parasitophorous vacuole within the host cell whereas micronemes are small vesicles that secrete proteins, participating in the recognition and adhesion to the host cell (Hemphill *et al*., 2013). The apical complex is set by conoid, polar rings,

micronemes and rhoptries (Dubey *et al.*, 1988a). The dense granules participate, by means of secretion products, both in the formation and in the maintenance of the parasitophorous vacuole inside the host cell (Hemphill, 1999). Certain differences in the arrangement and number of the secretory organelles are found between tachyzoite and bradyzoite stages (Speer & Dubey, 1989; Dubey *et al.,* 2004). It should be noted the presence of a vestigial apicoplast, present in most of apicomplexan parasites. This apicoplast have not photosynthetic capacity, but it has importance as a pharmacological target since it contains genetic material codifying for essential elements of the parasite's metabolism such as fatty acids synthesis (Sheiner *et al.*, 2013).

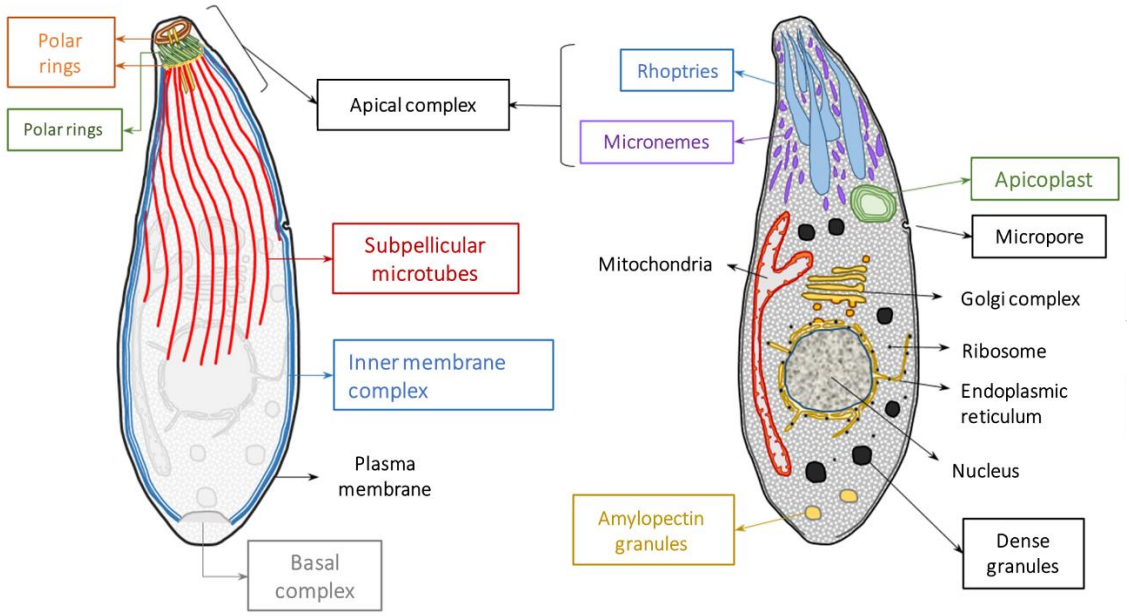


Figure 3. Graphic representation of the ultra-structure of the tachyzoite of *N. caninum*, with special mention of the cytoskeleton (left) and cytoplasm (right) components found only in apicomplexan protozoa. *Source Iván Pastor Fernández, Doctoral Thesis, 2015*

1.2 Life cycle and transmission

N. caninum is characterized by a complex facultative heteroxenous life cycle (Figure 4) that involves a definitive canid host and an intermediate host in which sexual and asexual replications take place, respectively. Up to date, the confirmed definitive hosts of *N. caninum* are the dog, the coyote, the dingo and the wolf. By resemblance to *T. gondii*, it is assumed that oocysts are generated in the intestinal tract of the definitive host after schizogony and gametogony and expelled in the faeces in an unsporulated (noninfectious) form. Oocysts become orally infectious after sporulation outside the host (reviewed by Dubey *et al.*, 2017).

The main intermediate host is cattle, although the exposure of a wide variety of domestic and wild animals to *N. caninum* has been shown in several studies. Viable tachyzoites have been isolated from the cow, the sheep, the water buffalo, the dog, the wolf, the horse, the bison and the white-tailed deer, although direct (parasite identification) or indirect (antibody responses) evidences of infection have been described in a range of other species. However, there is no evidence that *N. caninum* can infect humans because only low levels of antibodies have been

reported, and neither *N. caninum* DNA nor parasite presence has been demonstrated in human tissues (Dubey *et al.,* 2007; McCann *et al.,* 2008; Calero-Bernal *et al.,* 2019). After the ingestion of the oocysts, sporozoites are released in the gastrointestinal tract infecting the entero-epithelial cells. Inside, they switch to tachyzoites, which reach the bloodstream and produce the intra-organic spread of the parasite thanks to their ability to invade a wide range of organs and tissues. Finally, host immune responses lead to tachyzoite conversion, remaining the bradyzoites in immunopriviledged tissues (reviewed by Dubey *et al*., 2017).

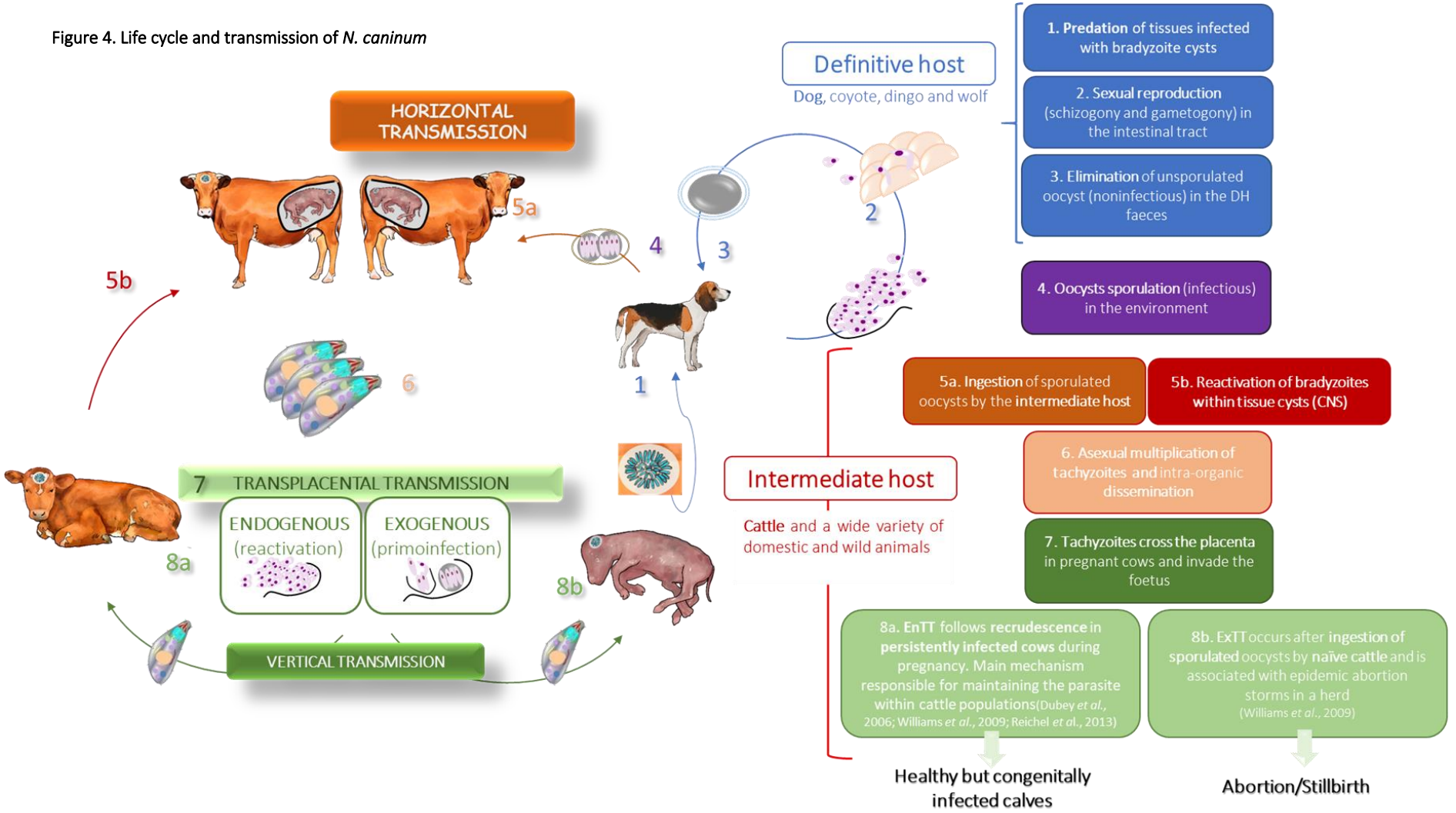
Predation of tissues infected with cysts containing bradyzoites is presented as the only way of transmission from the intermediate to the definitive host, thus closing the biological cycle. In the intermediate host, apart from the horizontal route, vertical or transplacental transmission can also occur when tachyzoites cross the placenta in a pregnant cow and invade the foetus, being the most efficient transmission route and playing a fundamental role in the propagation and maintenance of the disease in a herd (reviewed by Dubey *et al*., 2017).

Depending on the origin of the infection, two types of transplacental transmission are recognized in cattle: exogenous transplacental transmission (ExTT) and endogenous transplacental transmission (EnTT) (Trees & Williams, 2005). Pathogenic and epidemiological consequences as well as the control measures for both are different. ExTT occurs after ingestion of sporulated oocysts by naïve cattle and is associated with epidemic abortion storms in a herd (Williams *et al.,* 2009). EnTT follows recrudescence of infection in a persistently infected cow during pregnancy and is the main mechanism responsible for maintaining the parasite within cattle populations, resulting in foetal transmission rates as high as 95% (Dubey *et al.,* 2006; Williams *et al.,* 2009; Reichel *et al.,* 2013). Although vertical transmission of the pathogen predominates in cattle, some works have showed a decrease in the vertical transmission rates with age and absence of transplacental transmission in some cases, suggesting a higher importance of horizontal transmission than traditionally assumed (French *et al.,* 1999; Dubey *et al.,* 2007; Eiras *et al.,* 2011).

In this sense, EnTT appears as the predominant mode of transmission in many herds, while controversy exists regarding the relevance of ExTT to give rise to a chronic infection (McCann *et al.,* 2007; Dijkstra *et al*., 2008). To date, only ExTT has been achieved experimentally in pregnant cows after infection with tachyzoites or oocysts (Williams *et al.,* 2000; Innes *et al.,* 2001; McCann *et al.,* 2007). However, EnTT in postnatally infected animals has been observed in natural infections (Dijkstra *et al.,* 2008). In Spain, different epidemiological scenarios have been described in which ExTT and EnTT can occur independently or in combination (Rojo-Montejo *et al.,* 2009c).

Spite of the ingestion of sporulated *N. caninum* oocysts is the only demonstrated natural mode of infection in cattle after birth, other forms of transmission have been postulated. The presence of *N. caninum*-DNA in milk, including colostrum, has been demonstrated. However, there is no conclusive evidence that lactogenic transmission of *N. caninum* occurs in the nature. Venereal transmission may be possible, but unlikely, as evidenced recently. Finally, studies indicate that *N. caninum* life cycle is maintained in a domestic cycle between dogs and cattle (reviewed by Dubey *et al*., 2017).

Figure 4. Life cycle and transmission of *N. caninum*



Compelling evidence suggests a sylvatic cycle involving domestic and wild canids, and ruminant and herbivore species in North America (McAllister *et al.,* 1998; Barling *et al.,* 2000; Gondim *et al.,* 2004b; Gondim, 2006). In Europe, similar sylvatic cycles involving wild ruminants and canids seem plausible but currently little evidence exists to support this hypothesis.

1.3 Prevalence and economic impact

Differences in prevalence of *N. caninum* infection between countries and regions, and between different production systems were found. Despite the problems found for comparing available studies, the lowest seroprevalences in Europe were found in Sweden and Germany, whereas Holland and Spain showed moderate to high seroprevalences (Bartels *et al.,* 2006). South America, Argentina and Brazil presented the highest seroprevalences (80.9-97.2%) (reviewed by Dubey *et al*., 2017).

The first studies carried out in Spain showed a high herd prevalence, 55% and 83% in beef and dairy cattle, respectively, and individual prevalence values of 18% in beef and up to 36% in dairy cattle (Mainar-Jaime *et al.,* 1999; Quintanilla-Gozalo *et al.,* 1999). These data were similar to those found in later studies carried out in Galicia (northwestern region of Spain), where herd seroprevalences reached 88% in dairy cattle and 77% in beef cattle, and individual seroprevalences were stablished between 16% and 22% in dairy herds, and in 25% in beef herds (González-Warleta *et al.,* 2008; Eiras *et al.,* 2011).

In relation to abortion, between 12% and 40% of the aborted foetuses are infected by *N. caninum* worldwide (Dubey *et al.,* 2007). Two studies carried out in Spain revealed infection in 58% (González *et al.,* 1999) and in 39% of analyzed foetuses (Pereira-Bueno *et al.,* 2003), bringing to light the importance of *N. caninum* as an abortive agent in our country, even ahead of the virus of bovine viral diarrhea (BVDV).

Therefore, bovine neosporosis has been recognized as one of the main causes of reproductive failure worldwide, causing a negative economic impact on livestock production derived, directly, from the abortion and neonatal mortality and, indirectly, from the increase in the calving interval, the reduction of milk production and growth rate, the loss of genetic value of the animals and the increase of veterinary costs (reviewed by Dubey *et al*., 2017). Economic losses seem to be more important in dairy industry due to a higher risk of abortion. A recent study focused on the major livestock powers, Spain among them, estimated the impact of the disease at a billion dollars per year. National cost in Spain reached 19.8 and 9.8 million dollars in dairy and beef cattle, respectively (Reichel *et al.,* 2013).

1.4 Pathogenesis, clinical signs and lesions

Despite being one of the most important abortifacient agents in cattle, the final mechanisms triggered by *N. caninum* which lead to the foetal death are not completely understood. Regardless the origin of the infection, *N. caninum* tachyzoites replicate and disseminate throughout the host tissues, arriving to the placenta, where they can multiplicate, cross the maternal-foetal interface and reach the foetus (Dubey *et al.,* 2006). Four mechanisms have been

postulated so far as possible cause of abortion: tachyzoites multiplication in vital organs of the foetus; placental damage induced by parasite replication, with the consequent alteration of the placental functions; imbalance of the immune responses at the placenta; and prostaglandin secretion (Buxton *et al.,* 2002; Innes *et al.,* 2005; Innes, 2007).

While infection with *N. caninum* in non-pregnant cattle is generally asymptomatic, abortion is the most relevant clinical sign in pregnant animals. Abortion may occur from the third month of pregnancy, although it is usually observed between 5 and 6 months of gestation (Almería & López-Gatius, 2013). If infection occurs in the first third of gestation, the foetus is usually reabsorbed and heat repetition is observed. If the foetal death occurs between 3 and 8 months, the foetus is usually eliminated presenting moderate autolysis, although dead foetuses before the fifth month could be mummified and retained in the uterus for months. However, infection from the fifth month usually leads to the birth of healthy but congenitally infected calves that, generally, will present precolostral antibodies (Quintanilla-Gozalo *et al*. al., 2000; Williams *et al.,* 2000).

It is not very common the birth of very weak calves with neurological signs. When those appear, they may range from mild incoordination to complete paralysis. In severe cases, spine malformations, hydrocephalus or pneumonia can be observed. These signs usually appear in the first week after parturition, although they may appear until two weeks after birth and evolve to total paralysis and death of the animal during the first month of life (reviewed by Dubey *et al*., 2017).

Affected tissues of the host present microscopic lesions which facilitate the diagnosis of abortion. The lesions, mainly focused on placental and foetal tissues, are always inflammatory and non-suppurative. In the placenta, foci of necrosis and areas of intense inflammation with infiltration of mononuclear cells are usually observed, which, in advanced processes, can progress towards conjunctive regeneration with hyperplasia, fibrosis and even calcification of the necrotic foci (Barr *et al.,* 1994; Maley *et al.,* 2003). These infiltrations begin first in the maternal caruncles and then spread to the foetal cotyledons, with the appearance of areas of hemorrhage and necrosis. Separation of caruncle and cotyledon is often observed with release of serum into the maternal-foetal septum. In general, lesions are more severe when infection occurs earlier (Collantes-Fernández *et al.,* 2006a; Collantes-Fernández *et al.,* 2006c; Gibney *et al.,* 2008).

In foetuses with congenital infection, the lesions are mainly located in the CNS, with multifocal areas of lymphocytic infiltration, perivascular cuffs, microgliosis, presence of astrocytes and areas of necrosis (Barr *et al.,* 1991a; Dubey & Lindsay, 1996). Foci of non-suppurative inflammation have also been reported in the heart and in the liver, and, less frequently, in the kidney, the skeletal muscle and the lung. Interestingly, lesions were more severe when the abortion occurred after ExTT and during the first and second third of gestation (Dubey *et al.,* 2006; Collantes-Fernández *et al.,* 2006a; Collantes-Fernández *et al.,* 2006c). Congenitally infected calves with nervous signs usually have inflammatory lesions at the spinal level and the brain (Peters *et al.,* 2001). In contrast, the presence of lesions in congenitally infected calves without clinical signs and in adult animals is unusual and restricted to the CNS (reviewed by Dubey *et al*., 2017).

1.5 Diagnosis and control

- Diagnosis of neosporosis

Due to the lack of vaccines and effective treatments, the best control strategy for neosporosis is based on diagnosis. However, the diagnosis of bovine neosporosis is difficult and an adequate and systematic approach is needed. Serological testing of animals is sometimes necessary prior to entry into the herd or as a preliminary step to restocking. In abortion cases, epidemiological data and clinical history may suggest the involvement of *N. caninum* as a cause, but a correct laboratory diagnosis is always required. Currently, there is a large battery of diagnostic techniques, although not all them provide the same information or have the same reliability. Different strategies should be approached depending on the subject, and the choice of the most appropriate diagnostic tool for each case as well as its correct interpretation play a fundamental role (Ortega-Mora *et al.,* 2006).

Whether diagnosis is approached in foetuses, samples of the foetus (brain, heart, liver) and placenta are necessary to investigate histological lesions and presence of the parasite by polymerase chain reaction (PCR). The detection of specific or compatible lesions on target organs is of great value as an initial diagnostic approach and allows the association between the lesions and the presence of the parasite. However, the PCR provides greater sensitivity and specificity than immunohistochemistry to detect the parasite. Thoracic and abdominal fluids from foetuses older than 5 months are also useful for the determination of antibodies by indirect immunofluorescence (IFAT), enzyme-linked immunosorbent assay (ELISA) or Western blotting (WB), since antibodies from maternal origin cannot cross the placental barrier (Ortega-Mora *et al*., in press).

When diagnosis is carried out in individuals, serological diagnosis in both serum and milk samples can be used, which can also be analysed by IFAT, ELISA and WB. In order to demonstrate congenital transmission, selected samples should be precolostral serum or serum from calves older than six months, avoiding interference with colostral antibodies. If animals did not present abortion and purchase purposes are pursued, WB is the preferential technique since is more sensitive and specific. In these cases, sampling can be repeated after 4 weeks to observe seroconversion. After the abortion, there is an increase in antibody levels in many animals (Ortega-Mora *et al.,* in press).

Finally, if the objective is the serological diagnosis of the herd, the detection of antibodies in samples collected from the milk tank by ELISA is an optimal technique due to its low cost and speed. However, due to the dilution effect of the antibodies in the tank, it is only useful in herds with an intra-herd prevalence higher than 10-15%. Detection of antibodies in the milk tank is useful to know the initial state of the infection on a farm and its progress (surveillance). The detection of antibodies by ELISA or IFAT in aborted and unaborted cows allows the association of *N. caninum* infection with abortion by calculating the odds ratio. If the association between seropositivity and abortion is significant, we can ensure the involvement of neosporosis in abortion. The next step is to perform a serology on the different age groups on the farm. This information will be useful for the choice of the most effective control measures since we can have an idea of intra-herd seroprevalence, and we can also study the relationship between the seropositivity of related animals (mother-daughter) and know the predominant mode of

transmission within the herd. Finally, the avidity ELISA makes possible the differentiation of herds with a primoinfection and high risk of ExTT (low avidity in most animals) from herds with chronic infection and EnTT (high avidity) (Ortega-Mora *et al*., in press).

In addition, differential diagnosis with other causes of abortion, both transmissible and non-transmissible, must be performed.

- Control of *N. caninum* transmission

All the information obtained through the diagnosis protocol is important for the implementation of a control program in the herd, which is the only measure available for controlling bovine neosporosis since effective vaccines and treatments are not currently available, although they have been postulated as potential alternatives for the future. However, the cost-benefit ratio of the control measures, the economic losses caused by the infection and the epidemiological situation of each farm (seroprevalence and mode of transmission) must be considered when stablishing a control program (reviewed by Dubey *et al*., 2017).

  - Management measures

Farms with *N. caninum* free status must reinforce the biosecurity measures in order to maintain their sanitary status. Some basic biosecurity measures would include quarantine and serological analyses of new animals, control of dogs' access to the farms as well as other potential definitive hosts (wolf, fox), specially to food storage areas and feeding and drinking troughs, correct elimination of abortion wastes or animal tissues and control of wild reservoirs as rodents. Similarly, these practices should be implemented in those farms that have suffered an epidemic outbreak of abortions due to a primary infection and the consequent ExTT.

Farms where the infection is endemic must implement control measures focused on reducing EnTT. In farms with very low prevalence gradual selective culling of infected animals, starting with aborted cows, is recommended. When prevalence rates are intermediate or high, selective culling is economically unfeasible. In such cases, progressive elimination of the infection can be considered by culling of aborting cows and selective replacement with daughters of seronegative cows. The offspring of seropositive cows may be used to meat production (Hall *et al.,* 2005). In the case of seropositive animals of high genetic value, embryo transfer using seronegative cows as receptors or artificial insemination with meat breeds are possible alternatives (Dubey *et al.,* 2007; Reichel *et al.,* 2013).

In any case, serological monitoring at the herd level should be used as an indicator of the effectiveness of these control programs.

  - Vaccination

Vaccination against bovine neosporosis has proven to be the most effective control measure from an economic point of view (Reichel *et al.,* 2013). However, developing a vaccine designed to prevent both abortion and transplacental transmission supposes a big challenge. A commercial inactivated vaccine against bovine neosporosis (Bovilis NeoguardTM) was available for several

years in America and Oceania (Barling *et al.,* 2003). However, it was withdrawn from the market later, when some studies showed large differences in efficacy at the farm level and a lack of protection against vertical transmission (Weston *et al.,* 2012).

Live vaccination has delivered promising results and the best immune protection to date in experimental infections in cattle. However, there are still no commercial live vaccines since their large-scale production is difficult (Reichel *et al.,* 2015). Live vaccines include the use of *in vitro* attenuated isolates, genetically modified isolates or live isolates of low virulence such as Nc-Spain1H. On the other hand, inactivated vaccines, constituted by dead tachyzoites or antigenic extracts of the tachyzoites, have emerged as interesting alternatives to live vaccines, enhancing safety and stability of the final product and reducing costs. However, results in terms of protection against neosporosis have been negative or ambiguous (reviewed by Dubey *et al*., 2017).

Finally, new generation vaccines have been proposed as another long-term alternative, with advantages as immune response directed against specific targets or incorporation of specific markers that would differentiate between vaccinated and infected animals (DIVA) (Marugán-Hernández, 2017). Unfortunately, recombinant vaccines have shown very low efficacy compared to live vaccines *in vivo*. In this scenario, new approaches as the combination of different antigens, use of improved adjuvants and enhancers of the immune response (e.g. fusion to Toll-like receptor (TLR) ligands) or combinations of live parasites with new drugs are being tested (reviewed by Dubey *et al*., 2017).

- Chemotherapy and chemoprophylaxis

Although there is not an effective and safe treatment against neosporosis, recent research discovered promising compounds *in vitro* and *in vivo* (Müller & Hemphill, 2011). Diamido derivatives (Debache *et al.,* 2011; Schorer *et al.,* 2012), artemisinin (Mazuz *et al.,* 2012), miltefosine (Debache & Hemphill, 2012a), organometallic complexes of ruthenium (Barna *et al.,* 2013), inhibitors of protein kinase (BKIs) (Ojo *et al.,* 2014) or buparvaquone (Müller *et al.,* 2015) are some of these drugs. However, the greatest advances have been achieved with the use of some sulfadiazines, such as toltrazuril, which had some efficacy in experimental models, decreasing transplacental transmission in mice (Gottstein *et al.,* 2005; Strohbusch *et al.,* 2009). Despite these good results, there are some doubts regarding their practical use in natural infections, the possible presence of residues in products for human consumption and their high costs.

BKIs have been postulated as promising compounds against neosporosis and other apicomplexan parasites (reviewed by Dubey *et al*., 2017; Jiménez-Meléndez *et al.,* 2017). *In vitro* and *in vivo* models of *Neospora* demonstrated an inhibition of the viability and proliferation of the parasite (Ojo *et al.,* 2014), and partial protection against abortion and foetal dissemination in sheep (Sánchez-Sánchez *et al.,* 2018). The development of alternative formulations and the use of different routes of administration, drug dosages and dosing regimens have been proposed for improving their efficacy (Sánchez-Sánchez *et al.,* 2018).

2. Host-parasite interactions in bovine neosporosis: factors influencing abortion and transmission

Bovine neosporosis is one of the main causes of abortion in cattle. However, abortion is not the only consequence of the infection, the birth of weak calves or healthy but congenitally infected calves may also occur. As specified above, the specific causes determining the occurrence of abortion have not been completely elucidated yet, and different mechanism have been proposed as the possible triggers of abortion. However, the existence of factors influencing the transmission of the parasite and abortion have been previously demonstrated. These factors depend on the **host** and the **parasite**, being the most relevant the gestation period, the foetal and maternal immune responses and the transmission route as a part of host-dependent factors, and the parasite stage, the inoculation route, the dose of infection and the intraspecific variability of the *N. caninum* isolates as a part of parasite-dependent factors.

These factors have been investigated using both pregnant and non-pregnant mice and ruminant (bovine, sheep, goat and water buffalo) experimental models. For example, experimental infection in pregnant cattle at three different stages of gestation allowed to discern the influence of the gestation period in which the infection occurs by studying parasite distribution, parasite loads and lesions in aborted foetuses (Benavides *et al*., 2012; Bartley *et al*., 2012; Caspe *et al*., 2012; Bartley *et al*., 2013; Cantón *et al*., 2014a). Also, the influence of the breed, as another factor dependent on the host, has been studied by epidemiological surveys that suggested that certain breeds, mainly dairy (e.g. Holstein-Friesian), are more susceptible to infection than others (e.g. Limousin) (López-Gatius *et al*., 2005a; López-Gatius *et al*., 2005b; Armengol *et al.,* 2007). However, latest studies suggested that differences observed between breeds might have been caused by differences in the risk factors associated with the different production systems and not by differences in breed-related susceptibility to infection (Eiras *et al.,* 2011), although this must be experimentally studied. In addition to *in vivo* models, *in vitro* investigations have been useful to study the intraspecific variability of the isolates and new *-omics* techniques have increased the knowledge of the molecular basis of that virulence by means the comparative analyses of transcriptome, proteome and secretome, and have increased information on host-cell regulation events during infection between different virulence isolates.

2.1 Factors depending on the host

2.1.1 Gestation period

The time during gestation when infection occurs seems to be critical to the pregnancy outcome (Williams *et al.,* 2000; Innes, 2007). Experimental infections carried out within the **first trimester of pregnancy** (at 70 days of gestation) led to foetal loss generally associated with a multiplication or presence of the parasite in the placenta and the foetus, producing serious lesions that cause foetal death, with reabsorption, autolysis or mummification (Dubey *et al.,* 1992; Barr *et al.,* 1994; Williams *et al.,* 2000; Macaldowie *et al.,* 2004; Rojo-Montejo *et al.,* 2009a; Regidor-Cerrillo *et al.,* 2014). However, the birth of healthy calves without evidence of infection has also been described in some experimental infections using the subcutaneous (SC) route, thus a slower and controllable distribution of the parasite could be expected in this case, while other animals

infected with the same dose and isolate have aborted (Barr *et al.,* 1994, Williams *et al.,* 2000; Macaldowie *et al* . al., 2004; Gondim *et al.,* 2004c; McCann *et al.,* 2007; Caspe *et al.,* 2012).

Experimental infections carried out in the **second third of gestation** usually lead to the birth of healthy but congenitally infected calves. Sometimes neurological signs can be observed in the calf (Barr *et al.,* 1994; Innes *et al.,* 2001; Almería *et al.,* 2003; Gondim *et al.,* 2004c). Infection at mid-gestation can also cause abortion (Dubey *et al.,* 1992; Almería *et al.,* 2016; Vázquez *et al.,* submitted), although lesions during this period are less severe than those observed after infection in early pregnancy (Maley *et al.,* 2003). In fact, recent works using different parasite doses and administration routes at mid-gestation showed transplacental transmission and abortion (Vázquez *et al.,* submitted).

Finally, infection in the **last third of gestation** commonly leads to the birth of healthy calves although they are born congenitally infected (Barr *et al.,* 1994; Innes *et al.,* 2001; Williams *et al.,* 2000; Maley *et al.,* 2003; Cantón *et al.,* 2013). Moreover, lesions and parasite loads are lower than those found in infections carried out in the first and second third of gestation (Gibney *et al.,* 2008; Benavides *et al.,* 2012).

In **naturally infected cattle**, recrudescence of infection within the first half of gestation results in foetal death, whereas recrudescence later in gestation causes congenital infection but does not lead to the death of the foetus. However, most of naturally *Neospora*-induced abortions arise during mid-gestation, at five to six months (Dubey *et al.,* 2007; Almería & López-Gatius, 2013; Almería & López-Gatius, 2015). The dead foetus may be reabsorbed, may suffer autolysis or mummification or may be expelled before or during the parturition depending on the development of the foetus at the moment of the death (Dubey, 2005). However, in naturally infected cattle, the birth of infected and clinically healthy calves is the most frequent consequence of the infection, which suggests that transmission occurs mainly in late gestation (Quintanilla-Gozalo *et al.,* 2000; Pereira-Bueno *et al*., 2003).

Therefore, the outcome of the infection depends on the moment when infection or its reactivation takes place during gestation and it is related to maternal and foetal immune responses.

2.1.2 Foetal immune responses

The immunocompetence of the foetus at the time of the infection may determine its ability to control parasite infection and, therefore, its survival. The earlier the infection occurs during pregnancy, the more serious the consequences will be (Innes *et al.,* 2002; Collantes-Fernández *et al.,* 2006c). Bovine foetal immunocompetence develops gradually during the second half of pregnancy (Osburn *et al.,* 1982; Buxton *et al.,* 2002), recognizing and responding to pathogens during the second third of gestation. Therefore, foetus is very sensitive to infection during the first trimester of pregnancy (Williams *et al.,* 2000; Innes *et al.,* 2001), and the probability of foetal death is high. In the second third of gestation, the foetus can develop an immune response to infection although it might not be enough for its control (Williams *et al.,* 2000; Andrianarivo *et al.,* 2001; Almería *et al.,* 2003; Bartley *et al.,* 2004; Innes *et al.,* 2005). In the third trimester, the

foetus is already immunocompetent (Osburn *et al.,* 1982), controlling the multiplication of the parasite and limiting lesion development (Innes *et al.,* 2002; Dubey *et al.,* 2006). Experimental infections have supported this theory. Infection in early pregnancy was associated with parasite dissemination and *Neospora*-specific necrotic lesions, but no inflammatory reactions were detected in the foetuses (Macaldowie *et al.,* 2004; Gibney *et al.,* 2008), while infection in mid and late pregnancy resulted in fewer lesions and limited parasite dissemination (Maley *et al.,* 2003; Gibney *et al.,* 2008).

Based on these experimental results, it has been suggested that the immune response of the foetus is responsible for the outcome of the infection, rather than the maternal inflammatory response (Gibney *et al.,* 2008).

2.1.3 Maternal immune responses

Briefly, two components constitute the immune system: humoral immune system, consisting of antibodies produced by plasma cells derived from activated B lymphocytes, and cellular immune system, consisting of T lymphocytes, macrophages, natural killer (NK) and proteins secreted by them, cytokines and chemokines. When CD4+ T lymphocytes recognize antigens, expressed on the membrane of the antigen-presenting cells, the immune response is stimulated. CD4+ T lymphocytes are divided into two groups, T helper 1 (Th1) and T helper 2 (Th2), depending on the secreted cytokines. Cytokines and chemokines are the soluble mediators of the immune system, acting as a communication network between cells of both lymphoid and non-lymphoid origin (Entrican, 2002). These molecules can exert pleiotropic effects on their targets such as induction of cell migration between anatomical compartments, cell activation, cell proliferation and regulation of antibody production. Haematopoietic colony stimulating factors (CSFs), which induce leucocyte colony formation in the bone marrow, chemokines, which recruit these cells to anatomical sites where they are required to carry out their effector function, and interferons, that are part of the innate response effective at controlling certain virus and intracellular parasite infections, are also components of the immune response.

Th1 cells secrete pro-inflammatory cytokines IFN-γ, IL-12 and TNF-α stimulating the cell-mediated response, important for initiating immune responses that lead to the generation of T cells with a Th1-type cytokine profile. Th1-type responses are particularly effective at controlling intracellular infections, tumors and intracellular parasites as *Neospora*. On the other hand, Th2 cells secrete mainly IL-4, IL-10 and TGF-β, inhibiting the cell-mediated activity and increasing the humoral response (Entrican, 2002).

In non-pregnant animal, infection with *N. caninum* usually does not cause clinical disease since a combination of innate and acquired immunity mechanisms control the spread of the parasite by the organism (Innes *et al.,* 2001; Staska *et al.,* 2003; Almería *et al.,* 2003; Andrianarivo *et al.,* 2005; Rosbottom *et al.,* 2008; Bartley *et al.,* 2013; Regidor-Cerrillo *et al.,* 2014). IFN-γ produced by CD4+ T cells (Marks *et al*, 1998) limits multiplication of intracellular parasites (Innes *et al.,* 1995) and is an important component of protective immunity (Khan *et al.,* 1997; Baszler *et al.,* 1999). On the other hand, the role of TLR could be key during the initial phases of infection since they are present in antigen presenting cells (TLR-2), in B lymphocytes (TLR-9) (Werling *et al.,* 2006), and in

trophoblasts and other cell types within the placenta (Koga & Mor, 2008), recognizing pathogens and triggering innate immune responses against the parasite. Specifically, TLR-3, 7 and 8 have been recently implicated in *N. caninum* recognition in the bovine placenta (Marin *et al.,* 2017a; Marin *et al*., 2017b).

However, in the early stages of infection there is no clear trend towards cellular (Th1) or humoral (Th2) immune responses, since significant amounts of IFN-γ and IL-4 are secreted (Rosbottom *et al.,* 2007; Regidor-Cerrillo *et al.,* 2014). As the infection progresses, a polarization towards a Th1-type response occurs, predominating IFN-γ secretion over IL-4 secretion (Gibney *et al.,* 2008; Rojo-Montejo *et al.,* 2009a; Caspe *et al.,* 2012; Regidor-Cerrillo *et al*., 2014). IFN-γ activates CD4+ T cells, which stimulate CD8+ T cells, also capable of secreting IFN-γ (Maley *et al.,* 2006; Rosbottom *et al.,* 2007). This immune response eliminates most of infected cells, inhibiting the parasite proliferation through the secretion of IFN-γ and TNF-α and blocking the invasion of new cells through the antibodies generated against tachyzoites (Innes, 2007). In this process, the secretion of IL-17 by CD4+ T cells previously activated by macrophages also seems to play a key role in the control of the infection (Flynn & Marshall, 2011). The immune response described is capable of drastically reducing the number of tachyzoites disseminated by the organism, remaining only bradyzoites, which are located in immuno-privileged tissues.

Limited data from experimental infections suggested that when horizontal transmission occurs in non-pregnant animals, those animals might develop enough immunity to protect against vertical transmission during a subsequent pregnancy (Dubey & Lindsay, 1996; Ho *et al.,* 1997; Williams *et al.,* 2000; Innes *et al.,* 2001). It has been demonstrated that the route of transmission influences the maternal immune responses and the outcome of the infection. ExTT leads to lower vertical transmission rates than EnTT, and some animals are able to clear the infection (Dijkstra *et al.,* 2008). ExTT of *N. caninum* in cattle have been related with epidemic abortion, characterized by high parasite burden and high rates of abortion. However, immune responses generated during ExTT seem to confer protection against the parasite in successive gestations (Innes *et al.,* 2001; Williams *et al.,* 2003; Trees & Williams, 2005; McCann *et al.,* 2007), and there is some epidemiological evidence that this also happens in the field (McAllister *et al.,* 2000). On the other hand, there is no evidence, to our knowledge, that protective immunity to EnTT in *N. caninum* can occur. Moreover, recrudescence of a persistent infection during gestation have been observed (Williams *et al.,* 2003), and immune responses do not seem enough to avoid vertical transmission in successive gestations. Thus, the incidence of foetal infection and foetopathy in the population might be positively related to infection prevalence where EnTT is dominant, and negatively related to infection prevalence where only ExTT occurs (Williams & Trees, 2005).

Finally, infection with *N. caninum* during pregnancy or recrudescence leads to the parasite multiplication in the placenta, altering the immunological balance at the maternal-foetal interface with an increase of local pro-inflammatory IFN-γ, IL-12p40 and TNF-α cytokines which could compromise the gestation, together with an increase in IL-4 and IL-10 levels (Rosbottom *et al.,* 2008; Regidor-Cerrillo *et al.,* 2014), which avoids the immunological rejection of the foetus but favours the multiplication and vertical transmission of the parasite (Entrican, 2002; Innes *et al.,* 2002).

When infection occurs **early in gestation**, the immune system of the dam can mount a strong cell proliferation response with production of high levels of Th1 and Th2 cytokines at the maternal-foetal interface together with intense placental infiltration of CD4+ and γδ T lymphocytes, NK cells and a high production of local IFN-γ (Maley *et al.,* 2006; Rosbottom *et al.,* 2008; Regidor-Cerrillo *et al.,* 2014; Cantón *et al.,* 2014b). Therefore, it is assumed that the abortion may be caused by the presence of Th1 and Th2-type cytokines at the maternal-foetal interface causing intense inflammation and placental tissue damage as the maternal immune system fights the infection. This local immune response is not observed when the foetus survives (Maley *et al.,* 2006).

During gestation, especially in the **second third**, a significant downregulation of cellular response to mitogen, a reduction in cell proliferation in response to specific *N. caninum* antigens and a lower IFN-γ production is observed in the dam (Innes *et al.,* 2001). This fact is extremely important to avoid a possible immunological rejection of the foetus by the mother (Innes *et al.,* 2005) but, on the other hand, might be a trigger for recrudescence of infection in persistently infected cows, and the Th2 cytokine environment at the maternal-foetal interface would favour parasite invasion of the placenta and infection of the foetus.

Moreover, when cows were infected **late in gestation**, no abortion occurred and no lesions were observed in the foetuses, suggesting a control of the infection by the maternal -and foetal- immune responses (Benavides *et al.,* 2012). Although a high rate of vertical transmission was observed, parasites were observed later in the placenta, from 28 dpi onwards (Maley *et al.,* 2003; Macaldowie *et al.,* 2004). Thus, the immunomodulation of the dam in late pregnancy plays a role in limiting the initial infection (Benavides *et al.,* 2012), probably due to the specific production of IFN-γ by the mother at least partly (Rosbottom *et al.,* 2011).

Some studies postulated that difference in foetal death between early and late stages of pregnancy was more related to the intensity or magnitude of the immune response than to an imbalance of the Th1/Th2 responses (Rosbottom *et al.,* 2008). However, in one study an extensive inflammatory response characterized by an inflammatory infiltrate of CD4 + and CD8 + T cells and high levels of expression of IFN-γ and IL-4 was observed after the recrudescence of a natural infection in the second third of gestation (Rosbottom *et al.,* 2011), suggesting that this placental response, far from affecting foetal survival, could contribute to the parasitosis control. Therefore, it is not clear whether cytokines are beneficial or harmful to the host. It seems that it may depend on their concentration and location, as well as the gestation period. In this way, they could play an important role in the transplacental transmission of the parasite or mediate tissue damage, especially in the maternal-foetal interface.

2.1.4 Bovine placenta and neosporosis

As described above, many studies have been carried out in pregnant cattle. Results from those works indicated that the foetal-maternal interface is a key place where *N. caninum* replicates and has to cross in order to be transmitted to the progeny. The placenta is one of the most severely affected tissues, together with the foetal CNS (Dubey *et al.*, 2006). Microscopical lesions consisting of a non-suppurative inflammatory reaction in placenta have been widely described

(Barr *et al*., 1990; Macaldowie *et al*., 2004; Dubey *et al*., 2006; Cantón *et al*., 2014a). However, the pathogenesis of the infection at the maternal-foetal interface is not clear. Multiplication of the parasite within the placenta, with the subsequent tissue damage and alteration of the normal functions of the placenta have been proposed as mechanisms triggering the abortion. On the other hand, exacerbated immunological reactions in the placenta after infection with *N. caninum* is another hypothesis that could explain abortion (Innes, 2007). Be that as it may, primary infection with the parasite or reactivation lead to multiplication of the tachyzoites in host-tissues, arriving to the bloodstream and reaching the placenta, where the parasite preferentially replicates and cross the placental barrier, arriving to the foetus.

The placenta is a temporal, dynamic and diverse organ with important immunological features that facilitate embryonic and foetal development and survival. In addition, the polarized maternal and foetal cell layers conforming the maternal-foetal interface within the placentome function as a biological barrier, avoiding the passage of pathogens and toxic substances to the foetus. However, *N. caninum* is very effective at crossing this barrier. Studying the interactions between the parasite and the bovine placental host cells is necessary to understand the factors that precipitate abortion in some animals or allow transmission in others.

In order to understand the role that may play the placenta in the pathogenesis of the bovine neosporosis and the works carried out during the present Doctoral Thesis, some features of the bovine placenta are explained below. Among them, the placentome structure, summarized in Box 1, is one of the most important characteristics of the bovine placenta since it is the functional unit where the main functions take place.

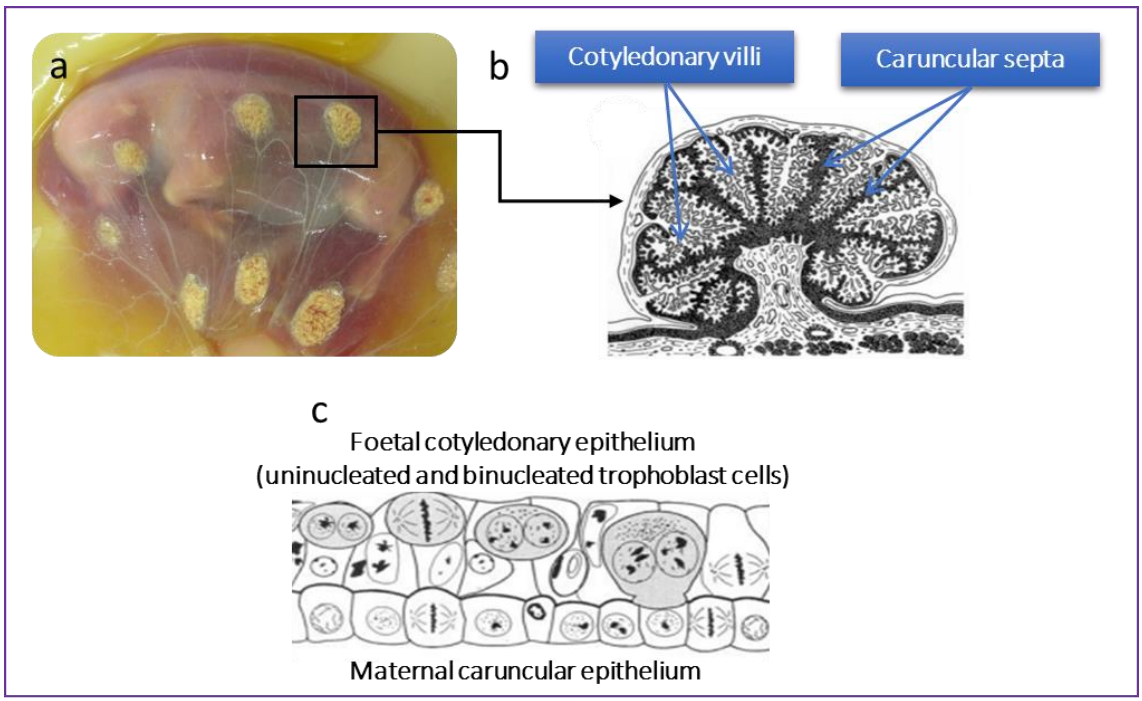


Figure 5. Representative images of the bovine placenta. (a) Image of a foetus within the placental membranes, (b) structure of bovine placentomes, and (c) main layers that conform the maternal-foetal interface.

Box 1: Placentome structure

The placentome is the primary area of the placenta where the exchange between the foetus and the dam occurs. The foetus absorbs oxygen and nutrients and excretes carbon dioxide and other wastes. Foetal cotyledons are interdigitated with maternal caruncles leading to **the formation of the functional units of the placenta, the placentomes** (Schlafer *et al.,* 2000) (Figure 5). The chorioallantoic membrane originates the cotyledon with villi that extend into crypts in the maternal epithelium. This intimate relation results in a strong interdigitation of the maternal and foetal tissues (Klisch *et al.,* 2010), forming placentomes characterized by a domed and ovoid mushroom-like structure (Laven & Peters, 2001), about 10-12 cm long and about 2-3 cm thick. By days 30-35 of gestation, three to four fragile attachments (early placentomes) are present in the pregnant horn; by day 40 attachments are present in both horns. At day 70, 40-50 placentomes are present, which will number 75-140 by mid-gestation. However, the contact between maternal and foetal tissues does not occur all along the uterus. The development of the cotyledons only occurs in receptive areas of the endometrium, the caruncles (Atkinson *et al.,* 1984). Caruncles are thickenings that appear early in the foetal uterus due to the proliferation of sub-epithelial connective tissue in the uterine mucosa (Schlafer *et al.,* 2000; Schmidt, 2005).

Although caruncles are present in the uterus before pregnancy, their development is a consequence of cotyledon development (Atkinson *et al.,* 1984). Placentomes are linked together by areas of flat apposition between trophoblast and glandular uterine epithelium (interplacentomal areas). Gland openings are covered by domes of phagocytic trophoblast (described below), which facilitate histotrophic uptake of endometrial gland secretions.

From the histological point of view, the placentome appears as a semi-placenta: each foetal villus consists of a structure of loose connective tissue, in which a tiny vessel from the foetus expands in a capillary network. That capillary network converges then in another vessel that arrives directly to the foetus. The surface of the villus is covered by monostratified epithelium. The maternal crypts are also covered by monostratified epithelium in which, by formation and fusion of syncytia, lacunae can arise (syndesmochorial placenta), and maternal vessels are divided into capillaries in the wall of the crypts. Therefore, in the bovine placenta there are no direct vascular connections. Within the placentome there is a migration and fusion of binucleated cells from the foetal compartment with maternal epithelial cells, leading to the temporal formation of syncytium (Figure 6).

The placentomes are connected on one side with the uterine wall and on the other side with the chorion. The connection with the uterine wall is produced through the peduncle of the caruncles, coated with uterine mucosa free of glands. The proliferation of the mucosa forms the dilated part of the caruncle, which, together with the peduncle, forms the maternal placenta. The peduncles of the caruncle, with a smooth and rounded surface, present small afferent and efferent vessels, originated from the uterine arteries. On the other hand, cotyledons develop in the chorioallantois, responsible for the connexion with the maternal placenta. The cotyledons surround the dilated part of the caruncles completely and only the insertion area of the peduncle remains free. Bovine placenta is classified as non-deciduate, as the maternal portion of the placenta is retained after parturition (Nickel & Seiferle, 2004).

Along the gestational period, the structures forming the placentomes suffer transformations in order to facilitate the transplacental exchange of nutrients, oxygen and waste products (Leiser *et al.,* 1997; Benirschke & Kaufmann, 2000). Placentome size may vary widely, from 5 to 15 cm in diameter. Weight and size of placentomes increase during gestation, particularly in the pregnant horn (Adeyinka, 2012).

In addition, placentomes in the pregnant horn are significantly larger than those in the non-pregnant horn (Laven & Peters, 2001). Placentomes are usually arranged in two dorsal and two ventral rows along both horns, although an extra row or reduced rows may be seen (Schlafer *et al.,* 2000). The number of placentomes varies widely between individual cows (Mossman, 1987; Laven & Peters, 2001), and later studies observed a trend towards an increase in the placentome number along the gestational period. However, the number of placentomes is important since abnormal placental development has been associated with a reduction in placentome number, which may be one cause for pregnancy loss (Lee *et al.,* 2004; Miglino *et al.,* 2007).

Therefore, placentomes are the interface between the maternal endometrium and the foetal trophoblast, being their normal growth fundamental for the establishment and maintenance of pregnancy and for normal foetal growth and development (Laven & Peters, 2001). In addition, placental steroidogenesis, fundamental not only for supporting the corpus luteum of pregnancy but also for the stimulation of placentome growth itself (Hoffmann & Schuler, 2002), takes place principally within placentomes. Hence, the placentome is a crucial component of the bovine placenta.

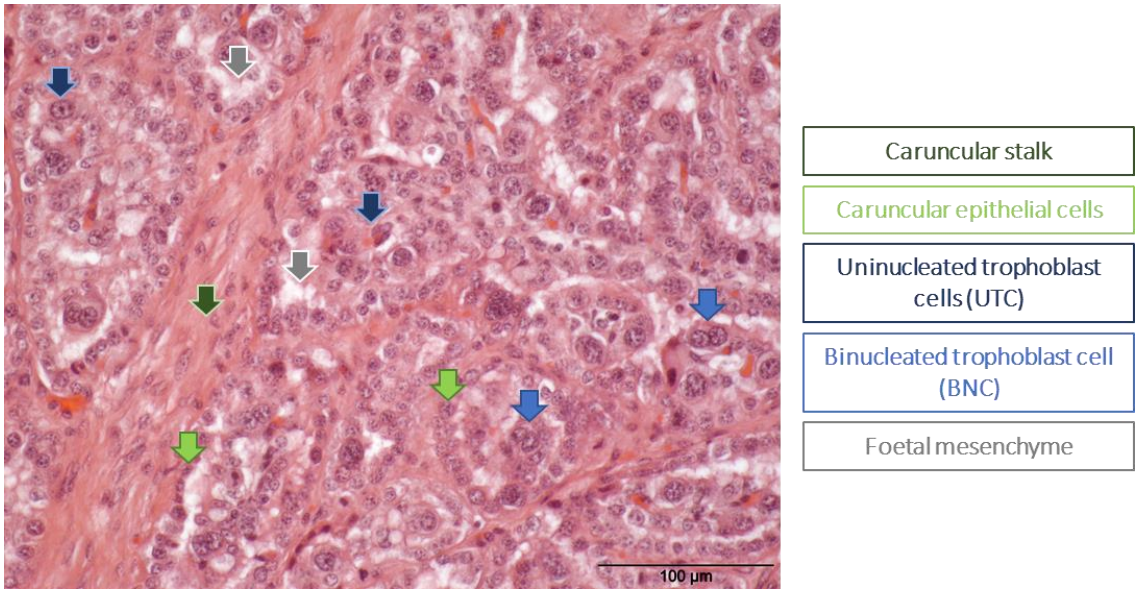


Figure 6. Representative image from histological slides of the bovine placenta stained with HE. The arrows indicate the most representative parts of the bovine placentome. 20×. Bar 100 µm

2.1.4.1 Trophoblast cells in the maternal-foetal interface

In general, the bovine trophoblast is composed of two populations of trophoblast cells, polarized uninucleated trophoblast cells (UTC) and trophoblast giant cells (TGC).

A unique feature of the synepitheliochorial bovine placenta are TGC, also named binucleated cells (BNC), which are the result of an acytokinetic mitosis (Klisch, 1999b). These cells are crucial for the feto-maternal interaction since they can migrate and fuse with maternal uterine epithelial cells, releasing a variety of products into the maternal compartment. However, nowadays the regulation of the processes of differentiation, genome multiplication, endocrine activities, migration and fusion of bovine binucleate cells are poorly understood.

Migration of BNC or TGC from the foetal trophectoderm into the maternal epithelium is crucial for the development of the placentome. After migration, fusion of trophoblast giant cells with single uterine epithelial cells leads to the formation of feto-maternal hybrid cells which are true syncitia in the maternal compartment of the placentome (Wimsatt, 1951; Wathes & Wooding, 1980). The migrating cells are mostly binucleated, non-polarized, arise from UTC by acytokinetic mitosis (Klisch *et al.,*1999a). They start to differentiate within the trophoblast at around 16 days of gestation (Wooding, 1992), just before implantation begins in cows (Björkman, 1969). BNC or TGC represent between 15 and 25% of all the chorionic epithelium during ruminant gestation (Wooding *et al*., 1997), with the remaining 80% consisting of UTC (Hradecky *et al.,* 1988). These BNC are distributed in a random manner throughout the trophoblast, persisting in the trophectoderm at a constant proportion from implantation up to one or two days before parturition, when there is a rapid decrease in their number (Wooding *et al.,* 1986). They are characterized by a dark-stained cytoplasm as a result of the presence of ribosomes and mature large Golgi bodies (Wango *et al.,* 1990), which produce large membrane bound granules occupying almost half of the volume of BNC (Rodriguez *et al.,* 2004).

Although BNC are present in every ruminant placenta, the uterine caruncular syncytium formed in cattle is different since in the cow the syncytium is displaced by maternal unicellular cells (Wooding & Wathes, 1980), degenerating (Wooding *et al*., 1997) and being mostly phagocytized by UTC (Klisch *et al.,* 1999b). Therefore, the syncytium formed by BNC is transient and the bovine placenta is classified as synepitheliochorial (Wooding, 1992). In addition, the migration of TGC does not continue beyond the maternal basement membrane and this migration has been described as “restricted trophoblast invasion/migration” (Pfarrer *et al.,* 2003).

In the placentome, there are small and scattered areas of local separation at these microvillar junctions, which contain maternal blood released intermittently from the maternal circulation. The tall columnar UTC of these separations actively participates in phagocytosis of erythrocytes, thereby providing iron for the foetus (Peter, 2013). In addition, UTC are involved in the production of interferon-τ (Roberts *et al.,* 1992).

BNC are not only unique because of their ability to migrate and their DNA content (most of them are tetraploid) (Klisch *et al.,* 1999b), but also due to the production of a variety of TGC specific glycoproteins and growth factors. After the BNC migrate and fuse with the maternal epithelium, they release their granules into the maternal tissue; these granules can be detected both in the maternal trinucleate cells and the syncytial places (Wooding & Wathes, 1980). These processes

of migration, fusion and granule release continue throughout gestation and are critical to the development of the conceptus (Wooding & Wathes, 1980).

The products of the BNCs include bovine placental lactogen (bPL), prolactin related protein-I (PRP-I) (Milosavljevic *et al.,* 1989), as well as, different pregnancy-associated glycoproteins (PAGs), which have been identified as potential moderators of interactions at the maternal-foetal interface and are expressed from implantation to term (Green *et al*., 2000; Klisch *et al*., 2005; Wooding *et al*., 2005). Recent studies have demonstrated that several growth factors like vascular endothelial growth factor (VEGF) (Pfarrer *et al.,* 2006b), platelet-activating factor (PAF) (Bücher *et al.,* 2006) and fibroblast growth factor (FGF) (Pfarrer *et al.,* 2006a) could be colocalized in TGC. These findings implicate that TGC survival, migration and fusion with uterine epithelial cells is a very complex process, and evidence that TGC are key regulators of various biological processes (angiogenesis, cell growth and differentiation and tissue remodelling) in the bovine placenta. Finally, TGC participate in the synthesis of steroids (Ullmann & Reimers, 1989; Matamoros *et al.,* 1994) and prostaglandins (Reimers *et al.,* 1985), essentials to support pregnancy. Furthermore, local steroid production by the placenta, particularly estrogens and progesterone, may also help to control caruncular growth, differentiation, and function (Hoffman & Schuler, 2002; Peter, 2013).

2.1.4.2 Extracellular matrix (ECM)

Formation of the placenta is a dynamic process that involves constant tissue remodelling, from implantation to delivery. Any tissue remodelling involves major changes/alterations of the ECM composition and relies on a delicate balance between matrix synthesis and degradation (Haeger *et al.,* 2016).

The ECM, which provides an environment for placental cells and regulates cell functions including signaling, proliferation, migration, and invasion, consists of a mixture of several unmodified and conjugated proteins such as proteoglycans and glycosaminoglycans and structural components such as several types of collagen that can be degraded by metalloproteinases (MMPs) (Kalluri & Zeisberg, 2006; Haeger *et al.,* 2016). Proteins representing the cytoskeleton (actin, desmin and vimentin), the ECM (collagen, fibronectin and laminin), and integrin receptors have been detected in bovine placenta using immunohistochemistry and can show a spatiotemporal expression pattern (Pfarrer *et al*., 2006; Zeiler *et al.,* 2007). Adhesion to the ECM in the bovine placenta is maintained by diverse proteins, including the adhesive transmembrane integrins, which are important receptors at the feto-maternal contact zone since they are bi-directional signal transducers (Haeger *et al.,* 2016). The role of cadherins in implantation and maternal-foetal communication was confirmed using cultured epithelial cells from bovine placentomes and caruncles (Bridger *et al*., 2008). The active interplay of ECM proteins during pregnancy ensures appropriate implantation, placental development, and timely placental detachment. Proteoglycans, such as decorin and biglycan, are small leucine-rich molecules responsible for fibrillogenesis, which expression patterns change over the course of pregnancy and differ between pathological and healthy placentas (Borbely *et al*., 2014). Decorin was isolated from the bovine placenta (Batbayar *et al*., 2000), and together with dermatopontin form a complex that may facilitate the binding of decorin to TGF-β1. Moreover, dermatopontin can bind to fibronectin.

Therefore, this complex may play a role in accelerating fibroblast adhesion and collagen fibrillogenesis, participating in tissue repair. It is logic then the downregulation of decorin observed during proliferation, remodelling and vascularization (Guillomot *et al*., 2014), dynamic processes that need ECM remodeling.

ECM molecules can be degraded by MMPs, zinc-dependent endopeptidases which comprise collagenases, gelatinases, matrilysins, and convertase-activatable MMPs (Fanjul-Fernández *et al.,* 2010). Their activity can be regulated by tissue inhibitors of MMP (TIMPs), chemokines, cytokines, and growth factors (Clark *et al.,* 2008). Both MMPs and TIMPs have been identified in bovine placentomes during physiological pregnancy contributing to placentome growth by degradation and remodelling of the ECM in both foetal and maternal compartments (Walter & Boos, 2001; Kizaki *et al.,* 2008; Dilly *et al.,* 2011), and under pathologic conditions. In fact, histochemical studies in bovine placentomes highlighted not only the MMP expression but also their activity and possible activators and inhibitors as factors that may be involved in mechanisms of physiological or pathological membrane separation (Walter & Boos, 2001). The MMP activity may be associated with the rigidity of the intravillous ECM within the bovine placenta (Franczyk *et al*., 2017), and MMP-2, 3, and 7 play an important role in the degradation of decorin. Among TIMPs, only TIMP-2 protein has been localized in bovine placentomes so far, concretely in TGC (Walter & Boos, 2001; Dilly *et al.,* 2011), whereas mRNAs from MMP-1, 3, 9, 13, and 16 and TIMP-1, 3, and 4 were increased in placentomes during calving (Dilly *et al.,* 2010; Dilly *et al*., 2011), suggesting their participation in placental remodelling.

Therefore, a multitude of functions of ECM and its components and modulators were suggested such as mediating trophoblast attachment to the uterine endometrium, inducing cell differentiation (Nakano *et al.,* 2002), being a reservoir of growth factors (Taipale & Keski-Oja, 1997), or serving as tracks on which cells can migrate along (Pfarrer *et al.,* 2003). However, implication of MMPs have also been observed in pathological conditions. Latest transcriptomic studies in bovine placentomes infected by *N. caninum* showed an upregulation of MMPs, specially MMP-13 (Horcajo *et al*., 2017), and MMPs might be involved in the crossing of *T. gondii* through the human placental barrier (Wang & Lai, 2013). In addition, MMPs from host infected with these apicomplexan parasites seem to be involved in infected macrophage dissemination in a mechanism resembling metastasis (Brasil *et al*., 2017), that may facilitate their transmission.

2.1.4.3 Foetal membranes and allantoic and amniotic fluids

The placenta is primarily made up of foetal membranes, which include the amnion, allantois, and chorion (collectively termed extraembryonic membranes). The inner membrane is the amnion, which is inverted around the embryo such that the ectoderm lines a cavity filled with amniotic liquor, composed by bronchial and salivary secretions of the foetus and a small part of urine, chlorate carbonate and sulfate of Ca, K and Na, creatine, creatinine, carbohydrates, albumin, variable amounts of mucin and foetal hairs, epithelial cells, epidermal desquamations or foetal faeces (meconium). The allantois, the intermediate membrane, is a secondary outgrowth of the extraembryonic splanchnopleure from the ventral hindgut. The mesoderm is vascular. The allantoic fluid is mainly made up of the urine of the foetus. Its pH is 6.5 to 7.5 and contains traces of uric acid, salts, creatine, allantoin and glucose. At the beginning of pregnancy, the

accumulation of fluids in the allantois distends the foetal membranes, which adhere to the uterine wall favoring nidation. In addition, foetal fluids have a protective role for the foetus, especially at the beginning of pregnancy, when the embryo is more fragile. Finally, the outer membrane, responsible for the interactions with the uterine endometrium, is the chorion.

The transitional stage between embryonic and foetal life is a phase of greater demand for the embryo. Therefore, greater activity of its membranes is required in order to establish relationships with the maternal organism. In this period, the vascularization of the allantois increases and the chorion fuses with the allantois, leading to the formation of allantochorion, that is the main component of the placenta. In fact, the foetal allantochorion adheres to the caruncles of the endometrium. Three layers are found in the allantochorial placenta: the endothelium, which line the allantoic blood vessels, the mesodermal connective tissue, and the chorionic epithelium, which derives from the trophoblast. In the uterine mucosa three layers can also be considered: the endothelium of the vessels, the adjacent connective tissue and the lining epithelium. Therefore, the allantoic epithelium is clearly involved in the transplacental exchange process (Leiser, 1975).

2.1.4.4 Placental functions

Placenta, with all its components, connects the foetus with the maternal uterus, providing nutrition and gaseous exchange. In bovine species, this connexion between mother and foetus occurs without direct contact between the foetal and maternal bloods (uterine-placental circulation). This organ acts as the digestive, respiratory, absorption, excretion, metabolic and endocrine systems of the foetus, allowing the passage of oxygen and nutritive principles from the mother to the foetus and catabolites from the foetus to the mother. The two umbilical arteries carry oxygenated (arterial) blood from the placenta to the foetus. Moreover, the placenta protects the foetus acting as a barrier or filter that prevents the passage of pathogens and toxic substances, and it is considered a supplementary and transient extra-foetal organ since also works as a gland of internal secretion and participates, along with the corpus luteum, in the maintenance of pregnancy. Concretely, the trophectoderm is the main part of the placenta, and it is remarkably versatile. It is characterized by a great capacity for invasion, cell fusion, hormone production, specific nutrient absorption, selective transport, active metabolism, and finally by its ability to resist maternal immunological attack.

Placental functions are detailed below and summarized in Figure 7.

2.1.4.4.1 Nutritive function

An epithelium-specific polarized morphology of both layers displaying apical microvilli and junctional complexes are characteristic for the maternal-foetal interface (Björkman, 1973; Leiser, 1975). The passive exchange of foetal and maternal metabolites is limited to small molecules only.

The nutrient can cross the bovine placenta by simple diffusion, facilitated diffusion or active transport. Low molecular weight compounds can pass through the placenta by simple diffusion according to the Donnan's effect, from the part with higher concentration to the part with lower concentration, until the equilibrium is reached in both parts. It is a very fast process and energy

is not consumed. Water, electrolytes, urea, uric acid, creatine, oxygen, CO_2 and some drugs are diffused by this mechanism. Other substances need to join a carrier in order to cross the placenta. It is also a very fast method and consumption of energy is not needed. Substances such as glucose, amino acids and fatty acids necessary for the foetus pass across the placental barrier using this mechanism. Finally, active transport allows the transit of complex compounds, which are divided into simple constituents and resynthesized after having crossed the placental membrane. Proteins, phospholipids and neutral fats go across the placental barrier by active transport with energy consumption.

In addition, the exchanges depend on the hemodynamic flows in the placentome and, consequently, are linked to the maternal hemodynamic situation, the foetal cardiac efficiency and the contractions of the uterine musculature.

2.1.4.4.2 Gas exchange across the placenta

The gaseous exchanges (O_2-CO_2) across the materno-foetal barrier depends on the qualitative characteristics of the exchange membrane (surface and thickness), the speed of blood circulation in the placenta, the O_2-CO_2 tensions present in maternal and foetal blood (concentration gradient), but above all, the characteristics and the amount of foetal hemoglobin. It should be noted that foetal blood has less affinity for CO_2 than maternal blood, favoring the passage of CO_2 from the foetus to the mother (Donnelly & Campling, 2016).

2.1.4.4.3 Endocrine function

The placenta works as an endocrine organ. It secretes both peptide and steroid hormones, which act to maintain pregnancy and to prepare for parturition and lactation (Donnelly & Campling, 2016).

During the peri-implantation period, from day 9 to day 24 of gestation approximately, UTC of the elongating conceptus secrete IFN-τ (Roberts, 2007; Ealy & Yang, 2009; Bazer *et al.,* 2015), which is considered the major pregnancy recognition signal in ruminants (Roberts *et al.,* 1992). Its biological actions on the endometrium have been widely investigated (Roberts, 2007; Spencer *et al.,* 2007; Spencer *et al.,* 2008) and the major roles for IFN-τ include the inhibition of uterine prostaglandin PGF2α secretion, therefore preventing luteolysis, and the modulation of the innate immune responses (Hansen, 2011). The anti-luteolytic activity of IFN-τ remain until the implantation of the embryo is completed. In the successive phases of gestation, the luteotrophic action is ensured by the placental hormones, concretely by bPL. The main peptide hormone secreted by the bovine placenta is bPL (Duello *et al*., 1986), which is produced by BNC and is important for the maintenance of pregnancy. The secretion occurs in the cotyledons, in the area of contact with the maternal caruncles and it is discharged into the maternal bloodstream.

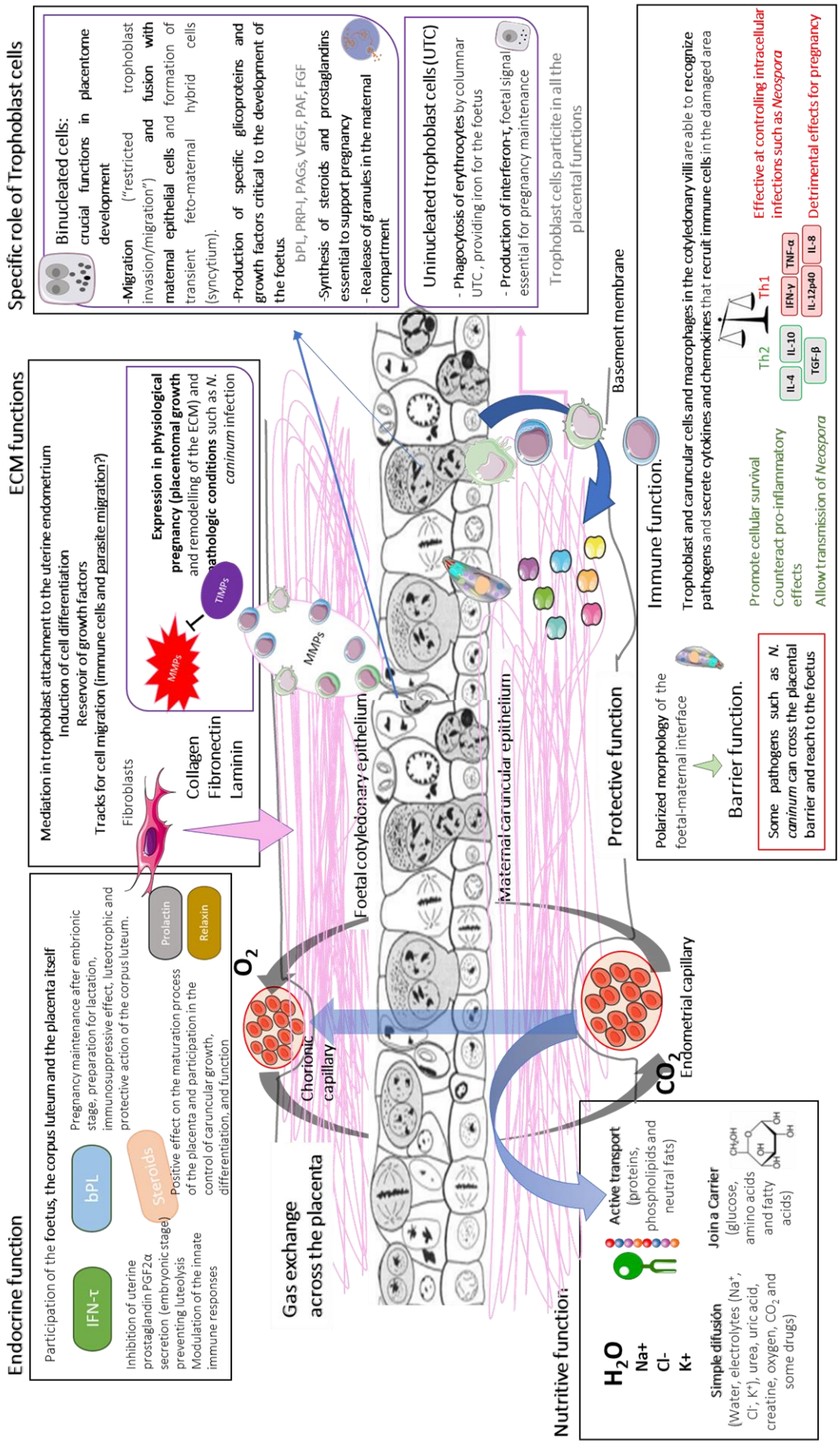


Figure 7. Graphical representation of the main placental functions

The role of the bPL includes many functions such as (i) preparation of the mammary gland for lactation; (ii) double effect in relation to insulin, stimulation of its production and secretion after the incorporation of carbohydrates or increase in peripheral resistance, mobilization of fats and glycogenolysis during fasting period; and (iii) immunosuppressive effect. The bPL together with the progesterone also has luteotrophic and protective action of the corpus luteum. The uterus under stimulation of the progesterone and bPL loses its tone, the wall becomes thinner and the blood supply increases.

To evaluate the steroid production of the placenta, it is necessary to consider the participation of the foetus, the corpus luteum, the placenta itself and the fundamental stages of steroid production for each hormone. Thus, placental activities are closely integrated with the foetal activities, coining the term "foetus-placental unit". The major steroids secreted by the placenta are progesterone and estrogens. The placenta is able, at the trophoblastic level (UTC and TGC), to produce progesterone in a remarkable amount and estrogens. Estrogens are believed to have a positive effect on the maturation process of the placenta (Grunert *et al.,* 1989). Local steroid production by the placenta, particularly estrogens and progesterone, may also help to control caruncular growth, differentiation and function (Hoffman & Schuler, 2002).

It is important to note that prolactin and relaxin are also produced by the placenta. Their concentrations in maternal blood increases at the end of gestation and during delivery preparing the mammary glands for lactation and relaxing the ligaments of the pelvic belt and intervertebral tail, as well as shortening and dilation of the cervix, respectively.

2.1.4.4.4 Protective function

The placenta is a protective barrier for the foetus against germs and drugs that circulate in the maternal blood. The polarized morphology of both layers that conform the maternal-foetal interface only allows the exchange of simple molecules. In fact, maternal antibodies, for example, cannot enter the foetal circulation, resulting necessary for the calf to receive colostrum within the first few hours after birth. However, viruses have, in relation to their small dimensions, the possibility of overcoming the placental filter in each period of gestation and their damage is related to the named embryogenetic calendar. In addition, some organisms, as *N. caninum*, can replicate within the cells conforming the maternal-foetal interface, the caruncular epithelial cells and the epithelial trophoblast cells, and pass across the placental barrier arriving to the foetus. In fact, *N. caninum* is one of the most efficiently transmitted pathogens by the transplacental route. Trophoblast and caruncular cells are able to recognize pathogens and secrete cytokines and chemokines that recruit immune cells in the damaged area (Montes *et al.,* 1995; Steinborn *et al.,* 1998a; Steinborn *et al*., 1998b). Moreover, an important increment of macrophages in the cotyledonary villi is observed in foetuses older than 6 months. These cells can produce proinflammatory cytokines and present antigens, suggesting that they work as sentinel cells, and hence they have an important role in foetal defence. On the other hand, it has been postulated than macrophages in foetal villi may also contribute to transplacental transmission of microbial agents (Schlafer *et al.,* 2000).

From the immunological point of view, gestation is considered a transplantation of immunologically foreign material, which must be tolerated by the mother. Therefore, physiological pregnancy is considered a Th2 phenomenon essentially supported by the cytokines

produced by Th2 lymphocytes. However, how the maternal immune system is able to tolerate the conceptus allowing the development of the foetus is not fully understood. In the last years, the Danger Model theory has offered an explanation for successful pregnancy. This theory states that placental structure is likely to influence the immunological features at the maternal-foetal interface in different species. The epitheliochorial bovine placenta is relatively non-invasive and the foetus would not suppose a risk for the mother, so the immune system of the dam does not react against the foetus. A danger signal would be necessary to activate the maternal immune system. Danger signals are tissue damage and destruction, distress and non-apoptotic cell death. Infection would constitute such a signal (Matzinger, 1994; Entrican, 2002).

Anyway, it seems clear that a balance of inflammatory and regulatory cytokines is important to ensure that the response itself is not damaging to the host by causing immunopathological effects. During the implantation phase, the predominantly lymphocytic population at the endometrium are NK cells, whose number increase in the luteal phase and throughout the first trimester of gestation. The NK cells are lymphocytes characterized by a cytotoxic ability and those presents in the decidua have been associated with the ability to interrupt gestation. The endometrium seems to have the capacity to inhibit the activity of NK, producing immunosuppressive factors, as progesterone, that favour implantation and gestation. The trophoblast also represents a barrier capable of resisting the attack of conventional cytotoxic T cells, thanks to the secretion of TGF-β, with local immunosuppressive effect. Under pathological conditions, there is a displacement of the cytokine equilibrium with prevalence of the Th1-type cytokines, especially IL-12, which activate NK, transforming them into powerful lymphokine-activated killer (LAK) cells capable of destroying the trophoblast by degranulation and releasing of proteolytic substances (perforins)(Elli, 2006).

Cytokines beneficial for pregnancy are IL-4, IL-10 and TGF-β (Innes *et al.,* 2007), as well as colony stimulating factors CSF-1 and GM-CSF, by inhibiting NK cell activity, by promoting trophoblast survival or by counteracting effects of inflammatory cytokines (Brach *et al.,* 1992; Irving & Lala, 1995; Chaouat *et al.,* 1995). Among those cytokines that appear to be detrimental are TNF-α, IFN-γ and IL-12, which are usually expressed at very low levels or are absent in normal placenta (Raghupathy, 1997; Roberston, 2000). TNF-α can cause thrombosis and smooth muscle contraction. It seems that TNF-α production by macrophages activates NK cells to produce IFN-γ, which further activates the macrophages to produce more TNF-α until the activated NK cells kill the embryo (Entrican, 2002). Finally, some cytokines may have both beneficial and detrimental effects, depending on concentration or stage of pregnancy (Innes *et al.,* 2005).

2.1.4.5 Cell cultures isolated from the bovine placenta

Decades of research into the molecular mechanisms by which the placenta forms and functions have sought to improve prevention, diagnosis, and management of disorders of this vital tissue. This research is quite advanced in human placenta. However, the placental tissues in different species reach their mature and anatomically distinct states *via* routes whose commonality of mechanism is often more assumed than confirmed (Huckle, 2017). Although differentiation of the early conceptus is very similar in human and cattle in size, the initial establishment with the uterine epithelium and subsequent growth and differentiation of the placenta are very different

between these two species (Schlafer *et al.*, 2000). Therefore, human placental cell cultures or explants do not seem the most adequate tool for the study of placental disease in cattle.

Bovine epithelial trophoblast and caruncular cell cultures are a convenient tool for a variety of future applications such as elucidation of the function of trophoblast-specific molecules that play a crucial role during pregnancy, clarification of the mechanisms that regulated the function of trophoblast-specific factors, learning about the cellular characteristics of caruncle and trophoblast, studying pathways of transport, communication and modification of metabolic substances, as well as studying pathways of infection of pregnancy associated diseases caused by parasites (e.g. *N. caninum*), bacteria (e. g. *Bacillus* spp.) and viruses (e.g. BVDV) or environmental toxins (e.g. plants, mycotoxins) (Bridger *et al.,* 2007b; Awad *et al.,* 2014). For these purposes, several cell lines have been established.

To develop a suitable *in vitro* model for both layers that conform the interface between the dam and the foetus, caruncular epithelial and trophoblast cells, has been a challenge in the last few years. While the isolation, characterization and long-term culture of caruncular epithelial cells was relatively easily achieved (Bridger *et al.,* 2007a; Bridger *et al*., 2007b), the establishment of a trophoblastic cell line resulted more complex and many attempts were made to culture bovine placental trophoblast cells (Figure 8).

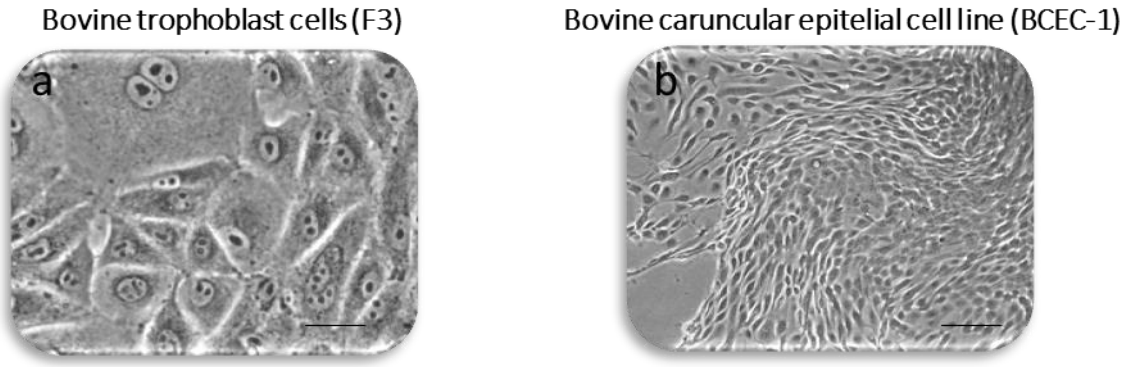


Figure 8. Representative images of bovine trophoblast cells (F3) (a) and bovine epithelial caruncular cells (BCEC-1) (b) in culture. (a) 20×. Bar 50 µm, (b) 10×. Bar 100 µm.

2.1.4.5.1 Bovine trophoblast cell cultures from cotyledon

Traditionally, the isolation of bovine trophoblast cells had two possible origins, cotyledons (Munson *et al.,* 1988; Vanselow *et al.,* 2008) or blastocysts (Talbot *et al.,* 2000; Shimada *et al.,* 2001) (Table 1). However, the success of these attempts has been limited, firstly because the trophoblast cell cultures turned out to be uncryopreservable, and secondly because the characterization of the isolated putative trophoblast cell cultures left doubts concerning the cellular origin.

The difficulties in the isolation of pure trophoblastic cell cultures from the cotyledon due to the heterogeneity of the tissue can be avoided when using non-implanted blastocysts as a source for trophoblast cells (Talbot *et al.,* 2000; Shimada *et al.,* 2001). However, blastocyst-derived cells are less differentiated and may not possess all properties characteristic for the cotyledonary trophoblast. Therefore, these cultures from pre-implanted blastocysts are useful for studying pre-

implantation and peri-implantation processes and early embryogenesis, but not for investigating processes that occur later during gestation. For example, abortions caused by *N. caninum* are more frequent during the second trimester of gestation in natural conditions, so the study of the pathways of *N. caninum* infection during transplacental transmission, as well as the evaluation of critical factors involved in placental pathogenesis should be done in trophoblast and caruncular cells isolated from caruncles and cotyledons, the real target for the parasite.

It was in the year 2010 when Hambruch and collaborators achieved the isolation and characterization of trophoblast cells from cotyledons of 5 months pregnant cows, obtaining the first establishment of a long-term stable bovine cotyledonary trophoblast cell line (F3) of confirmed origin. This cell culture presented a good growth efficiency and the cells maintained their characteristics of origin beyond 45 passages. In addition, trophoblast cells were preserved by freezing, overcoming the problems to freeze and low efficiency when passing found in other trophoblast cultures previously isolated. The obtained cell line showed epithelial morphology and characteristic BNC in small numbers through all passages. The trophoblastic origin of F3 cells was verified by amplification of a Y-chromosome specific DNA-sequence and the presence of bPL mRNA. Immunofluorescence demonstrated that F3 cells were continuously positive for zonula occludens-2, cytokeratin and vimentin. In conclusion, the F3 cell line showed several *in vivo* characteristics of bovine cotyledonary trophoblast cells. Several investigations have been approached using this cell line (Dilly *et al*., 2010; Haeger *et al*., 2011; Sobotta *et al*., 2017; Horcajo *et al*., 2017; Loch *et al*., 2018).

Following the methodology described by Hambruch *et al*. (2010), different research groups have obtained, characterized and used bovine trophoblast cell lines from cotyledonary origin (Martino *et al.,* 2015). Other authors isolated only BNC by Percoll gradient and infected them with *N. caninum* NC-1, demonstrating the phagocyte capacity of these cells and suggesting that BNC could potentially participate in the transplacental infection of bovine neosporosis (Machado *et al.,* 2007). In the last years, several research groups have continued the generation of trophoblast cells using new methodologies (Suzuki *et al.,* 2011; Kawaguchi *et al.,* 2016) (Table 1).

2.1.4.5.2 Epithelial cell cultures from bovine caruncle

Most of *in vitro* studies focussed on the physiology and pathology of reproduction in cows used primary bovine endometrial cell from non-pregnant animals, and seldom differentiated between caruncular and intercaruncular regions (Fortier *et al.,* 1988; Horn *et al.,* 1998; Kimmins *et al.,* 2003), which results fundamental since epithelia differ from each other functionally (Banu *et al.,* 2005; Pfarrer *et al.,* 2006a; Pfarrer *et al.,* 2006b). Only one bovine endometrial (epithelial) cell line (BEND) originating from a non-pregnant animal had been characterized and deposited at the American Type Culture Collection (ATCC) (Johnson *et al.,* 1999).

Cell cultures derived from the caruncle of pregnant animals have rarely been established (Table 1). The group of the professor Pfarrer from the University of Veterinary Medicine of Hannover, in the attempt to create a cell culture model to study foetal-maternal interactions, established and validated a method to isolate and cultivate primary epithelial cells from the caruncle. The resultant cell line, named BCEC-1 (bovine caruncular epithelial cell line-1), was isolated from a pregnant cow (*Bos taurus*) with an estimated gestational age of 4 months (Bridger *et al.,* 2007a)

(Table 1). For characterization of bovine epithelial cells, criteria such as phase contrast microscopy (Munson *et al.,* 1991; Bridger *et al.,* 2007b), cytokeratin expression (Yamauchi *et al.,* 2003; Bridger *et al.,* 2007a), and measurement of transepithelial electrical resistance (Bridger *et al.,* 2007b) were established (Haeger *et al.,* 2016). BCEC-1 cells were proven to be of maternal origin, and it was demonstrated that they preserved their epithelial specific properties. Cell morphology, protein expression and the ability to form an intact epithelial barrier was maintained for at least 32 passages. Furthermore, cryopreservation of the cells was achieved. This cell line, was created in order to standardise future experimental conditions, representing a functional, differentiated caruncular epithelium from pregnant cows, and since its isolation some investigations using this cell line have been carried out (Bridger *et al*., 2008; Waterkotte *et al*., 2011; Sobotta *et al*., 2017).

The actual *in vivo* involvement of F3 and BCEC-1 during pregnancy offer a variety of future applications such as the study of pathways of infection of pregnancy associated diseases caused by parasites (e.g. *N. caninum*). In fact, these cells constitute a polarised cell culture model derived from the tissue participating in vertical transmission in pregnant cattle, so they are appropriate in order to study the interactions between different isolates of *N. caninum* and the bovine placenta during gestation.

Table 1. Summary of bovine trophoblast cultures isolated from blastocysts, cotyledons or by new methods and bovine caruncular cell cultures isolated from pregnant animals

Origin	Cell line	Reference	Scope
Blastocyst	BE-13	Stringfellow *et al.,* 1987	Establish a method by which trophectodermal cells originating from individual preimplantation embryos could be perpetuated in culture
	CT-1	Talbot *et al.,* 2000	Isolation and characterization of blastocyst-derived cell lines (CT-1 and CT-5)
	BT-1	Shimada *et al.,* 2001	Establishment of a bovine trophoblast-1 cell line (BT-1), derived from *in vitro* matured and fertilized blastocyst; in the absence of feeder cells
	-	Ramos-Ibeas *et al.,* 2014	Description of an efficient method to obtain a trophoblastic cell line from a single bovine embryo or from an embryo biopsy
	-	Pillai *et al.,* 2019	Blastocyst-derived trophoblast
Cotyledon	-	Munson *et al.,* 1988	Isolation and culture of bovine cotyledonary trophoblastic cells from early second trimester placentas by collagenase digestion
	-	Bridger *et al.,* 2007a	Isolation and validation of primary epitheloid cell cultures from bovine placental caruncles and cotyledons
	-	Vanselow *et al.,* 2008	Establishment and evaluation of a tissue culture model for bovine trophoblasts by collagenase digestion
	F3	**Hambruch *et al.,* 2010**	Isolation and characterization of a bovine trophoblast cell line F3
	Line A Line B	Martino *et al.,* 2015	Involvement of the Kiss-1R/Kps system in regulating trophoblast invasion and proliferation in bovine primary placenta cotyledon cell cultures
New methods	BT-(A-L)	Suzuki *et al.,* 2011	Establishment of 12 bovine trophoblastic cell lines using bone morphogenetic protein 4 (BMP4)
	biTBCs	Kawaguchi *et al.,* 2016	Generation of trophoblast cell lines (biTBCs) from bovine amnion-derived cells (bADCs) using an induced pluripotent stem cell technique
Caruncle	Several caruncular cells	Bridger *et al.,* 2007a	Isolation and validation of primary epitheloid cell cultures from bovine placental caruncles and cotyledons
	BCEC-1	**Bridger *et al.,* 2007b**	Establishment of an epithelial cell line from caruncles of pregnant cows and development of a model to study restricted trophoblast invasion, pathogenesis of pregnancy associated diseases and pathways of infection and transport

2.2 Factors depending on the parasite

2.2.1 Parasite stage, inoculation route and dose of infection

Most of the experimental studies on ruminants have been carried out with tachyzoites maintained *in vitro* since the infectious parasite stage for ruminants in natural infection, the oocyst, is difficult to obtain. Some authors have reproduced the infection by oral administration of sporulated oocysts (Trees *et al.,* 2002; Gondim *et al.,* 2004c; McCann *et al.,* 2007), although the rates of abortion described were significantly lower than those observed in studies performed by parenteral inoculation of tachyzoites. The establishment of the infection and the transplacental transmission depended on the dose administered and the stage of gestation when oocysts were used (Trees *et al*., 2002; Gondim *et al*., 2004c; McCann *et al*., 2007). However, comparisons between different studies using oocysts are difficult because of the variability in the viability of oocysts (reviewed by Dubey *et al*., 2017).

Due to their susceptibility to digestion, *N. caninum* tachyzoites have usually been inoculated through a parenteral route (SC, intravenous -IV- or intramuscular -IM-). The IV and SC routes have been the most assayed routes of inoculation and IV route is associated with a more severe and reproducible clinical presentation in terms of abortion compared with the SC route (Macaldowie *et al*., 2004). However, some authors have suggested that SC inoculation may model the natural infection better than IV inoculation, since it allows the parasite to be processed through the lymph nodes before being distributed through blood circulation (Dubey & Schares, 2006). On the other hand, the effect of the infection dose using tachyzoites as inocula is related to the route of administration and the host species. In cattle, IV doses have ranged from $1\text{-}2\times10^6$ tachyzoites (Weber *et al*., 2013) to 5×10^8 (Macaldowie *et al*., 2004), causing infection in all inoculated dams at day 76 of gestation and 75% of abortions, and both infection and abortion in all cows infected on day 70 of gestation, respectively. Recently, the administration of higher doses of the parasite have also been associated with higher rates of abortion and the development of more severe tissue lesions in a pregnant bovine model infected at mid-gestation (Vázquez *et al.,* submitted). SC inoculation of cattle with doses of 10^7 and 5×10^8 tachyzoites caused transplacental transmission and lesions in the foetus when inoculated in the second trimester of gestation, although the higher dose was associated with earlier and more severe histopathological changes (Maley *et al*., 2003). The IM route was used in the first studies of experimental infections in cattle (Dubey *et al.,* 1992), and later in combination with IV or SC inoculations (Conrad *et al.,* 1993b; Barr *et al.,* 1994; Andrianarivo *et al.,* 2000; Andrianarivo *et al*., 2001). Other pathways such as conjunctival have been sporadically used, resulting in infection and induction of a specific immune response in pregnant cattle without transplacental transmission (de Yaniz *et al.,* 2007; Moore *et al.,* 2014). Finally, the possibility of venereal transmission of neosporosis has been investigated. Experimental studies have shown that heifers are highly susceptible to intrauterine inoculation of semen spiked with tachyzoites (Serrano-Martínez *et al*., 2007a; Serrano-Martínez *et al*., 2007b), whereas adult cows were less susceptible to becoming infected using this route of inoculation (Canada *et al*., 2006; Serrano-Martínez *et al*., 2007b).

Therefore, the vast majority of studies use IV or SC routes and the majority of experimental infections have been performed with very high doses, demonstrating that IV inoculation led to

more serious consequences for the foetus and that higher doses of parasite are related to higher abortion rate and more severe lesions.

2.2.2 Isolates and intraspecific variability

As indicated above, one of the most important factors influencing abortion and transmission directly related to the parasite is the intraspecific variability of *N. caninum* isolates, which has been widely demonstrated both *in vivo* and *in vitro*. An extensive variation in virulence was demonstrated in *N. caninum*, which may be associated with variation in the clinical presentation of the disease, although little is known about the strain variation with respect to pathogenicity.

To date, more than 100 *N. caninum* isolates have been obtained from domestic animals (cattle, water buffalo, sheep and dog) and wild animals (white-tailed deer, European bison and wolf) (Ortega-Mora *et al*., in press). At first, isolation of *N. caninum* focused on severe cases of the disease, for example, isolates NC-1 and Nc-Liverpool (Nc-Liv) were obtained from the encephalon of dogs with clinical signs, whereas the isolate Nc-SweB1 was obtained from the brain of an aborted bovine foetus (Dubey *et al.,* 1988b; Barber *et al*., 1995; Stenlund *et al.,* 1997). In Spain, SALUVET research group has obtained 10 isolates, mainly from congenitally infected and clinically healthy calves, with the aim of studying isolates with differences in the behaviour (Regidor-Cerrillo *et al.,* 2008; Rojo-Montejo *et al.,* 2009b).

The availability of these isolates has made possible their comparison, facilitating the study of the intra-specific variability of the parasite. There are not specific animal models suitable for testing strain variation, and a meaningful comparison with pregnant cattle would be economically and ethically prohibitive. However, intra-specific variability of *N. caninum* isolates has been approached in the last years using both *in vitro* (cell culture techniques) and *in vivo* models (murine and ruminant models). Up to date, several authors have shown differences in the invasion and proliferation (Schock *et al.,* 2001; Regidor-Cerrillo *et al.,* 2011; Dellarupe *et al.,* 2014b; García-Sánchez *et al.,* 2019), as well as in the dissemination and transmigration abilities *in vitro* (Collantes-Fernández *et al.,* 2012; García-Sánchez *et al.,* 2019). In addition, the characterization of a large population of bovine and canine isolates in mice demonstrated that some isolates were more virulent and more efficiently transmitted to the progeny than others (Lindsay *et al.,* 1995; Atkinson *et al.,* 1999; Collantes-Fernández *et al.,* 2006b; Rojo-Montejo *et al.,* 2009b; Regidor-Cerrillo *et al.,* 2010; Dellarupe *et al.,* 2014a), and recent results appeared to indicate that these findings in mice could reflect the effect of different isolates in cattle (Rojo-Montejo *et al.,* 2009a; Caspe *et al.,* 2012; Regidor-Cerrillo *et al.,* 2014; Almería *et al.,* 2016a).

The knowledge obtained from those comparisons between isolates opens up new revenues for research and may allow the identification of new virulence factors, which is essential for the development of pharmacological and immunoprophylactic tools. Nowadays, the proliferating molecular approaches are presented as very useful tools for the study of intraspecific variability of the parasite.

2.2.2.1 Phenotypic variability in *in vitro* models

According to the National Research Council of USA, experimental models are a critical component in biomedical research. They include animals, cells and all kind of cultures, as well as computational and mathematical simulations to improve human and animal health. On the other hand, the scientific world is increasingly aware of the welfare and ethics of animal experimentation. In this sense, since the publication of *The Principles of Humane Experimental Technique (*Russell & Burch, 1959), scientists and government agencies have endorsed replacement, reduction, and refinement as essential tools for promoting the humane treatment of research animals. Replacement alternatives refer to methods that avoid or substitute the use of animals. *In vivo* procedures should be replaced whenever possible by alternative methods that do not use animals, such as mathematical models or *in vitro* biological systems. Reduction is referred to any strategy that consists of using a smaller number of animals with scientifically valid results, or the maximization of the information obtained per animal. The refinement of the experimental design and the selection of the most appropriate model contribute to the fulfilment of this principle. Finally, refinement alternatives include all procedures to minimize or eliminate pain, as well as all enrichment methods to ensure animal welfare.

In this context, the use of *in vitro* cultures has been widely accepted in order to provide an initial approach to certain mechanisms involved in complex processes since they allow the obtaining of reproducible results and the reduction of experimental animals as well as the optimization of the methods that will be used later during *in vivo* experiments. It has been demonstrated that *in vitro* models have numerous applications and, concretely, *in vitro* culture systems represent powerful tools for the study of apicomplexan parasites (Muller &Hemphill, 2012). In addition, tachyzoites can be maintained in cell culture by continuous passage. However, several contradictory studies indicated that tachyzoites cultured for a long number of passes showed a diminished pathogenicity in the murine model (Long *et al.,* 1998; Bartley *et al.,* 2006) or adaptation to cell culture (Pérez-Zaballos *et al.,* 2005). Be that as it may, prolongated culture of tachyzoites seems to alter their original characteristics.

One of the most important aplications of *in vitro* models in the research of apicomplexan parasites has been the understaining of the processes implicated in the lytic cycle of these parasites. The lytic cycle of *N. caninum* and other intracellular apicomplexan parasites comprises the processes of adhesion and invasion of the host cell, adaptation to intracellular conditions, maturation of the parasitophorous vacuole and multiplication of the parasite and, finally, egression of the tachyzoites from the vacuole causing the lysis of the host cell (Figure 9).This sequence of events is required for parasite survival and propagation in the course of animal infection (Smith, 1995; Black & Boothroyd, 2000). All these processes are strictly regulated by the sequential secretion of proteins by different organelles of the parasite such as rhoptries (ROP), micronemes (MIC) and dense granules (GRA), which interact directly with the host cell and modulate multiple functions, favouring the intracellular multiplication of the parasite. These processes have been more widely investigated in the zoonotic agent *T. gondii* at the molecular level (Dowse & Soldati, 2004; Carruthers & Boothroyd, 2007; Lim *et al.,* 2012), but it is assumed that they may be similar in *N. caninum* due to the existence of conserved proteins with a high degree of homology (Hemphill *et al.,* 2006). It has been demonstrated that *N. caninum* tachyzoites are able of invading and multiplying in a wide variety of cell types (Hemphill *et al.,*

2004; Pérez-Zaballos *et al.,* 2005; Hemphill *et al*., 2006; Müller & Hemphill, 2013), from bovine monocytes and endothelial cells (Lindsay & Dubey, 1989) to stable cell lines as kidney cells, human fibroblasts (HFF) or cells derived from the kidney of the African green monkey (Vero, MARC-145), being the latest the most used (Hemphill *et al.,* 1999; Risco-Castillo *et al.,* 2004; Regidor-Cerrillo *et al.,* 2011).

In addition to lytic cycle studies, *in vitro* models have been used with different aims. Next, we will focus on the studies of isolate characterization and the existing heterogeneity of the *N. caninum* isolates. In this sense, several studies have revealed that *N. caninum* isolates differ in certain *in vitro* phenotypes related to virulence such as tachyzoite invasion and growth rate, tachyzoite dissemination and transmigration through biological barriers and immune response modulation or evasion (Saeij *et al.,* 2005; Taylor *et al.,* 2006; Lambert & Barragan, 2010; Pollar *et al.,* 2012). These phenotypic traits are key in the *N. caninum* virulence and may directly influence dynamics and outcome of infection in the natural host.

In vitro works focussed on the comparison of different isolates carried out up to date are discussed below.

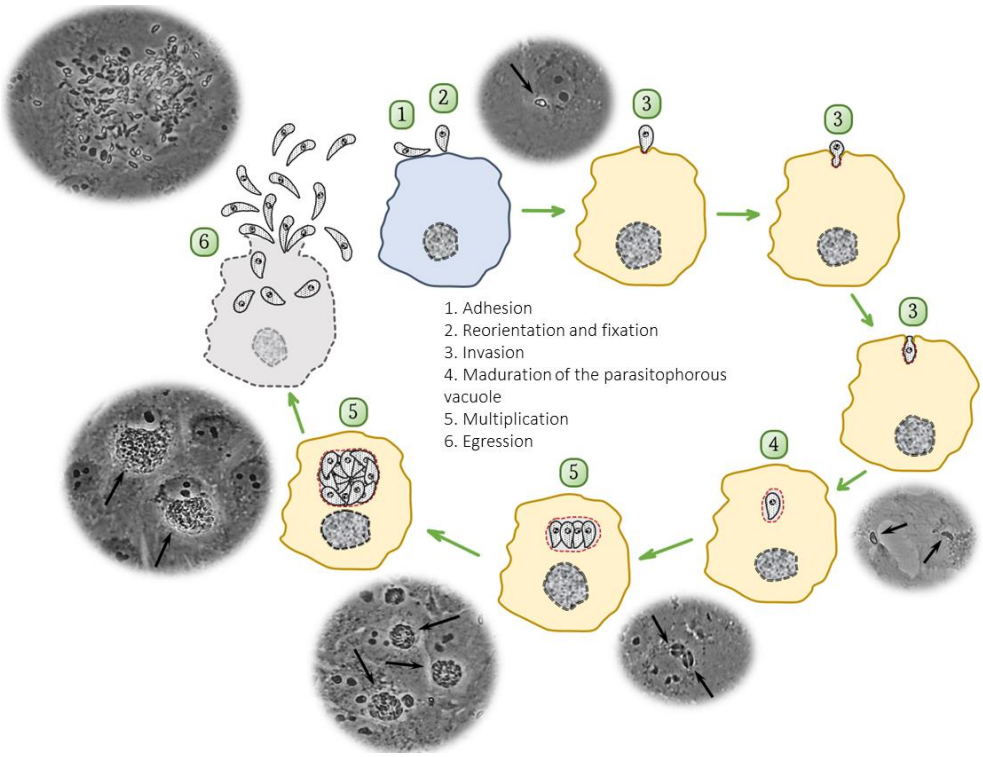


Figure 9. Graphic representation of the lytic cycle of *N. caninum* and the image in cell culture. Source *David Arranz Solís, Doctoral Thesis, 2016*

• Tachyzoite invasion and growth. High invasion, replication and tachyzoite yield *in vitro* was correlated with faster proliferation of the parasite in host tissues, reaching high parasite burdens in short time periods, which cause severe tissue damage and abortion. An extensive variability in the tachyzoite growth of *N. caninum* isolates have been demonstrated (Schock *et al.,* 2001; Rojo-Montejo *et al.,* 2009a; Regidor-Cerrillo *et al.,* 2011; Dellarupe *et al*., 2014b), and results are summarized in Table 2. In addition, a significant correlation between tachyzoite yield and pup mortality was reported in a BALB/c pregnant model for neosporosis (Regidor-Cerrillo *et al.,* 2010; Dellarupe *et al.,* 2014a), which supports the hypothesis that isolates with the highest inherent proliferation capacities reach higher parasite burdens in target host-tissues (foetal and placental), contributing to host tissue damage and clinical signs. However, a limitation of this kind of studies is the adaptation of tachyzoites to the *in vitro* culture, modifying its *in vivo* biological behaviour and virulence (Pérez-Zaballos *et al.,* 2005; Bartley *et al.,* 2008), which makes necessary the use of parasites with a low number of passages in cell culture (Regidor-Cerrillo *et al.,* 2011; Dellarupe *et al.,* 2014b).

• Migration. Higher migration capacities have been associated with wider parasite dissemination as well as with easier access to immunopriviledged sites, such as the CNS or the foetus. In *T. gondii* there is evidence of two putative complementary pathways utilized for crossing restrictive cellular barriers. *T. gondii* clonal genotype I tachyzoites exhibit a transmigratory ability in epithelial cell monolayers, linked to parasite gliding motility and virulence in mice (Barragan & Sibley*,* 2002; Taylor *et al.,* 2006) whereas genotypes II and III induce a potent hypermigratory phenotype in DC, which have been identified as systemic carriers (Trojan horses) of *T. gondii* tachyzoites (Lambert *et al.,* 2009; Lambert *et al.,* 2011). These dissemination strategies seem to be conserved among coccidian apicomplexan parasites, since a similar isolate-specific migratory pattern was observed for *N. canimum* in human DC (Collantes-Fernández *et al.,* 2012). Some *N. caninum* isolates exhibited a higher dependency on DC-mediated transmigration whereas other isolates exhibited a relatively higher dependency on extracellular tachyzoite transmigration for efficient translocation across polarized BeWo monolayers *in vitro*. Recently, Nc-Spain7-infected bovine macrophages showed a significantly higher hypermotility than those infected with Nc-Spain1H (García-Sánchez *et al.,* 2019).

• Parasite interaction with the host immune system. The ability of *T. gondii* to interact with the host cell through virulence factors and its correlation with subversion or induction of protective immune responses have been described in a high number of studies (Saeij *et al.,* 2005; Blader & Saeij *et al.,* 2009; Melo *et al.,* 2013). This ability is directly related to strain virulence. Macrophages infected with type I and III strains are alternatively activated through phosphorylated STAT6 due to their polymorphic rhoptry kinase ROP16, while type II infected macrophages are classically activated through activating of NF-κB by the dense granule protein GRA15 (Jensen *et al*., 2011). However, the interaction of *N. caninum* with host cell signalling, especially with immune cells, have been barely studied. Studies in murine macrophages recognized TLR2-MAPK, TLR3-TRIF, TLR11-MEK/ERK pathways and NLRP3-inflammasome activation to be implicated in host-resistance against *N. caninum* by inducing production of proinflammatory cytokines IL-12p40, IL-1β, IL-6, TNF-α and IFN-γ (Jin *et al.,* 2017; Li *et al.,* 2018; Wang *et al.,* 2018). Mota and collaborators (2016) demonstrated that p38 phosphorylation was quickly triggered in murine macrophages stimulated by live tachyzoites, while its chemical inhibition resulted in upregulation of IL-12p40 production and augmented B7/MHC costimulatory

molecules expression, showing that *N. caninum* manipulates p38 phosphorylation in its benefit, in order to downregulate the host's innate immune responses. A recent work studied the infection of *N. caninum* NC-1 isolate in human monocyte-derived macrophages (THP-1 cells), showing that *N. caninum* infection produced increased expression of pro-inflammatory cytokines (TNFα, IL-1β, IL-8, IFNγ) as well as induced expression of host-defence peptides (cathelicidins) mediated by MEK 1/2. Secretion of cytokines and cathelicidins from *N. caninum*-infected human macrophages reduced parasite internalization and promoted the secretion of pro-inflammatory cytokines in naïve macrophages (Boucher *et al.,* 2018). Although these studies provide important advances, remarkable differences regarding the immune response between mice and human and bovine species exist, including the lack of TLR11 and TLR12 in the genome of cattle, among others (Jungi *et al.,* 2011). In addition, the previous works did not compare different *N. caninum* isolates and, therefore, they did not demonstrate the possible heterogenicity of *N. caninum* isolates in immune response modulation. Thus, with the aim of determine the ability of two *N. caninum* isolates, which showed marked differences *in vivo* and *in vitro*, to modulate innate immune responses in the bovine host, SALUVET group has recently characterized for the first time *N. caninum* replication in bovine monocyte-derived macrophages and detailed isolate-dependent differences in host cell responses to the isolates Nc-Spain7 and Nc-Spain1H (García-Sánchez *et al.,* 2019). Although both isolates survived and replicated in bovine macrophages, Nc-Spain1H presented a delayed replication and a lower growth rate without an exponential growth pattern respect to Nc-Spain7, which was accompanied by higher reactive oxygen species (ROS) production, higher IL12p40 expression by Nc-Spain1H-infected macrophages, and higher IFN-γ release by lymphocytes than Nc-Spain7-infected cells. Furthermore, infected macrophages expressed higher levels of IL-10 and lower expression of MHC Class II, CD86 and CD1b molecules than uninfected macrophages, with non-significant differences between isolates. Recent transcriptome studies of infected bovine macrophages have confirmed capacity of *N. caninum* infection to induce a pro-inflammatory gene expression profile that varied according to the virulence of Nc-Spain1H and Nc-Spain7 isolates (García-Sánchez *et al.,* submitted).

In spite of the advances in the last years, little is known about the mechanisms used by *N. caninum* to cross the placenta and the factors that enable some isolates to be more effectively transmitted and cause foetal death than others.

2.2.2.2 Variability in the murine model

There is a great heterogeneity of murine models described in the literature. So far, the non-pregnant or brain infection model (Collantes-Fernández *et al.,* 2006b; Pereira García-Melo *et al.,* 2010) and the pregnant or congenital infection model (López-Pérez *et al.,* 2006; López-Pérez *et al.,* 2008) are the most commonly used. The main advantage of the brain infection model is the ease of handling, and a great utility in the chronic phase of infection. The parameters evaluated include morbidity and mortality, detection and parasite burden in the CNS, and the humoral and cellular immune responses. On the other hand, the pregnant mouse model is useful in the study of congenital neosporosis, and it has been presented as a more appropriate alternative than the non-pregnant model in the evaluation of the safety and efficacy of drugs and vaccines (Aguado-Martínez *et al.,* 2009; Marugán-Hernández *et al.,* 2011b; Rojo-Montejo *et al.,* 2011; Monney *et al.,* 2012; Debache & Hemphill, 2013). In addition to parameters evaluated in the non-pregnant

model, pregnant murine models allow the evaluation of cytokines at the placental level in mothers and the foetal death, morbidity, post-natal mortality and vertical transmission in the offspring. However, pregnant mice models present some disadvantages such as the synchronization of the estrus by the Whitten effect (Whitten, 1957), and a correct diagnosis of gestation. To date, most models of congenital neosporosis in mice emulate the ExTT, inducing the primary infection during pregnancy (Liddell *et al.,* 1999; Quinn *et al.,* 2002a; López-Pérez *et al.,* 2006; López-Pérez *et al.,* 2008), whereas attempts to reproduce EnTT have not been satisfactory (Jiménez-Ruiz *et al.,* 2013).

Numerous studies have been carried out with the aim of revealing aspects related to the pathogenesis of the disease (Table 2), the study of the immune response (Tanaka *et al.,* 2000; Nishikawa *et al.,* 2001; Ritter *et al.,* 2002; Viera da Silva *et al.,* 2017; He *et al.,* 2017), as well as for the evaluation of vaccine and pharmacological candidates (Cannas *et al.,* 2003a; Cannas *et al.,* 2003b; Ramamoorthy *et al.,* 2006; Aguado-Martínez *et al.,* 2009; Marugán-Hernández *et al.,* 2011b; Debache & Hemphill, 2012a; Debache & Hemphill, 2012b; Rojo-Montejo *et al.,* 2012; Pastor-Fernández *et al.,* 2015).

Table 2. Virulence classification of different isolates of *N. caninum* according to their behaviour in murine pregnant models and *in vitro*

Isolate	Origin, species (Reference)	Pregnant mice model					*In vitro* model		Virulence consensus
		Maternal morbidity	Parasite presence in maternal tissues	Neonatal morbidity	Neonatal mortality	Vertical transmission	$pInvR_{4h}$	TY_{56h}	
NC-1	Brain, dog with neurological signs (Dubey *et* al., 1988b)	Low	High	High	High 76.8%	High 92.8%	-	Mild	High
Nc-6Arg	Faeces (oocysts), dog (Basso *et al*., 2001)	Low	High	Low	Low 0%	Mild 53.8%	Mild	Mild	Low-Mild
Nc-Bahia	Dogs with neurological signs (Gondim *et al*., 2001)	High	High	High	High 100%	High 100%	High	High	High
Nc-Ger2	Faeces (oocysts), dog (Schares *et al*., 2005)	Low	Low	Low	Low 7.7%	Low 30.8%	Low	Low	Low
Nc-Ger3	Faeces (oocysts), dog (Schares *et al*., 2005)	Low	Mild	Mild	Mild 19%	Low 9.1%	Mild	High	Mild
Nc-Ger6	Faeces (oocysts), dog (Schares *et al.*, 2005)	Low	Low	Low	Low 4%	Low 23.1%	Low	Low	Low
Nc-Liverpool	Brain, dog with neurological signs (Barber *et al*., 1995)	High	High	High	High 100%	High 100%	High	High	High
Nc-Nowra	Brain, asymptomatic but congenitally infected calf (Miller *et al*., 2002)	Low	Low	Mild	Low	High 87%	-	-	Low
Nc-Spain1H	Brain, asymptomatic but congenitally infected calf (Rojo-Montejo *et al*., 2009a)	Low	Low	Low	Low 0.5%	Low 5%	Low	Low	Low
Nc-Spain2H	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	Low	Low	Mild	Mild 20%	Mild 61.3%	Mild	Low	Low-Mild
Nc-Spain3H	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	Low	Mild	Low	Low 5%	High 89%	Low	Low	Low-Mild
Nc-Spain4H	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	High	High	High	High 100%	High 97.3%	High	High	High
Nc-Spain5H	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	High	High	High	High 95%	High 100%	Mild	High	High
Nc-Spain6	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	Low	Low	Mild	Mild 30%	Mild 57.6%	Mild	High	Low-Mild
Nc-Spain7	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	High	Mild	High	High 95%	High 79.1%	Mild	High	High
Nc-Spain8	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	Low	Low	Low	Low 1.1%	Mild 56.4%	Mild	Low	Low-Mild
Nc-Spain9	Brain, asymptomatic but congenitally infected calf (Regidor-Cerrillo *et al*., 2008)	Low	Low	Mild	Mild 30%	Mild 52.6%	Mild	Mild	Low-Mild
Nc-Spain10	Brain, congenitally infected calf with clinical signs (Regidor-Cerrillo *et al*., 2008)	Low	Mild	Mild	Mild 18-20%	Mild 65.5%	Mild	High	Mild
Nc-SweB1	Brain, aborted calf (Atkinson *et al*., 1999)	Mild	Mild	-	Low	-	-	Low	Mild

Atkinson *et al*., 1999; Miller *et al*., 2002; Quinn *et al*., 2002a; Collantes-Fernández *et al*., 2006b; Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2010; Pereira García-Melo *et al*., 2010; Regidor-Cerrillo *et al*., 2011; Jiménez-Ruiz *et al*., 2013; Dellarupe *et al*., 2014a; Dellarupe *et al*., 2014b

2.2.2.3 Variability in the bovine model

The bovine model, as target species of neosporosis, is the ideal model to study the consequences of parasite infection (Benavides *et al.,* 2014). This model will be useful to study host-pathogen interactions and host immunity at the local and systemic level, as well as for evaluating diagnostics, vaccines and therapeutics. Both pregnant and non-pregnant models have been developed in cattle (Benavides *et al.,* 2014). However, a standardized bovine model has not been established yet since there is a great diversity of experimental conditions employed, which influence the result of the infection, therefore resulting in a high level of variability and a trouble for comparison between experiments. These factors, previously exposed, include the gestation period, breed, parasite isolate and stage, and dose and route of inoculation of the parasite (Benavides *et al.,* 2014). Thus, standardization is needed to advance research in a more collaborative, timely and efficient manner.

2.2.2.3.1 Non-pregnant bovine model

Most of the studies performed in non-pregnant ruminants aimed to conduct research on the parasite cycle or the possibility of post-natal infection (Benavides *et al.,* 2014). Cyclical oral transmission between dog and cattle was proven using this model (Gondim *et al*. 2002). The possibility of post-natal transmission of neosporosis has been addressed in new-born calves. It was demonstrated that tachyzoites present in colostrum could transmit the infection, although the infection has not been reproduced in calves (Davison *et al.,* 2001). The possibility of venereal transmission of the disease have also been investigated using bovine experimental models. *N. caninum* DNA was detected in the semen of infected bulls (Ortega-Mora *et al.,* 2003), although venereal transmission has not been demonstrated yet (Ferre *et al.,* 2008; Osoro *et al.,* 2009). Finally, numerous experimental studies have been carried out to characterize the immune responses occurring after *N. caninum* infection (Conrad *et al.,* 1993b; Marks *et al*., 1998; Lunden *et al*., 1998; De Marez *et al*., 1999; Maley *et al*., 2001).

2.2.2.3.2 Pregnant bovine model

As occurred in the murine models, primoinfection models, valid for evaluating ExTT, are the most common and their main objective is the evaluation of abortion and transplacental transmission. However, EnTT has not been experimentally reproduced yet (McCann *et al.,* 2007; Benavides *et al.,* 2014).

- Foetal mortality model:

Foetal mortality has been induced efficiently by IV inoculation of a high number of tachyzoites (10^7 - 5×10^8) at 70 days of gestation, principally from NC-1, Nc-Liv and Nc-Spain7 isolates. Parameters such as clinical observation (foetal viability and fever), detection of parasites (spread of the parasite in different organs of the dam and the foetus), presence of lesions in the foetus and in the placenta, and study of the immune responses (systemic humoral and cellular immune

responses as well as local expression of cytokines in the placenta or maternal tissues) have been used to monitor foetal death and transmission.

However, the model of foetal mortality do not seem the most adequate to represent the natural infection since *Neospora* associated abortions have been described mostly at mid-gestation in the field (Dubey *et al.,* 2007). In addition, IV infection on day 70 with the doses usually used could be excessively aggressive to carry out a correct evaluation of the efficacy of drugs or vaccines, underestimating some potentially effective products (Benavides *et al.,* 2014), and to establish *N. caninum* isolate virulence (Regidor-Cerrillo *et al*., 2014).

- Vertical transmission model:

In this model, the infection is carried out from the second third of gestation (Williams *et al.,* 2000; Innes *et al.,* 2001; Almería *et al.,* 2003; De Yaniz *et al.,* 2007; Rosbottom *et al.,* 2007; Wiengcharoen *et al.,* 2011; Rojo-Montejo *et al.,* 2013; Almería *et al.,* 2016a; Almería *et al.,* 2016b). The same parameters described for the foetal death model are valid to monitor the characteristics and consequences of the infection of this model, considering that here the most important thing is to determine whether transplacental transmission has occurred or not. Therefore, techniques are usually applied for detection of specific precolostral antibodies (ELISA, IFAT), determination of parasite presence (PCR, IHC) and observation of compatible lesions in several tissues (histology). In addition, other analyses can provide additional information. For example, in live vaccine assays, the genotyping of *N. caninum* by microsatellites analysis in foetal tissues allows to discriminate the infection by the isolate used in the vaccine or in the challenge (Rojo-Montejo *et al.,* 2013).

The bovine models are very useful for the study of the pathogenesis of the infection and the developed immune response, both peripheral and local in the placenta and the foetus. Numerous studies have investigated the distribution of the parasite, the lesions and the type of immune response (innate and memory) that occurs after infection, both in non-pregnant and pregnant cows (Benavides *et al.,* 2014). The role of TLR (Bartley *et al.,* 2013), as well as the humoral (Williams *et al.,* 2000; Bartley *et al.,* 2004; Macaldowie *et al.,* 2004; Serrano *et al.,* 2006; Rojo-Montejo *et al.,* 2009a; Rojo-Montejo *et al.,* 2013; Regidor-Cerrillo *et al.,* 2014) and cellular responses, highlighting CD4+ and CD8+ T lymphocytes (Marks *et al.,* 1998; Staska *et al.,* 2003; Tuo *et al.,* 2005; Rosbottom *et al.,* 2007; Rocchi *et al.,* 2011) and cytokines at the placental level (Rosbottom *et al.,* 2008; Rosbottom *et al.,* 2011; Almería *et al.,* 2011; Regidor-Cerrillo *et al.,* 2014; Cantón *et al.,* 2014b; Almería *et al.,* 2016a; Darwich *et al.,* 2016), among others, have been studied. Recently, the lectin-binding pattern in the placentas of cows infected experimentally with *N. caninum* was studied by IHC techniques (Dorsch *et al.,* 2019), proving the importance of the changes occurred in the ECM during *N. caninum* infection. Bovine models have also been used to evaluate the efficacy and safety of pharmacological treatments and vaccines in their final phase of development (Andrianarivo *et al.,* 1999; Kritzner *et al.,* 2002; Miller *et al.,* 2002; Williams *et al.,* 2007; Baszler *et al.,* 2008; Vanleeuwen *et al.,* 2011; Moore *et al.,* 2011; Hecker *et al.,* 2013; Rojo-Montejo *et al.,* 2013; Weber *et al*., 2013).

Finally, bovine models have been used to assess the biological diversity of *N. caninum*. Differences between isolates previously characterized in the murine model have been demonstrated in the

bovine model. In the numerous experimental infections carried out in pregnant ruminants, several *N. caninum* isolates have been employed. However, the comparison of those isolates is difficult as the experimental design varied considerably among the studies, as commented above. In addition, economic and infrastructure constraints involved in experimental infection of large animals are also impediments for developing this type of studies (Benavides *et al.,* 2014). For these reasons, few studies comparing isolates in bovine models have been carried out. In these studies, the Nc-Spain1H isolate (low-virulent isolate that failed to induce mortality and led to a low transplacental transmission in mice), did not damage the foetus after infection at an early stage of gestation, whereas NC-1 caused death in 3 of 5 foetuses (Rojo-Montejo *et al.,* 2009b). Furthermore, more severe lesions as well as higher frequency of parasite detection in placental and foetal tissues from NC-1 infected animals were found. In a similar comparative study, the Nc-Bahia isolate showed lower pathogenicity than NC-1 isolate in cows and buffaloes (Chryssafidis *et al*., 2014). Although in both inoculated groups there was a high vertical transmission, lesions observed in NC-1 infected group were more severe, and the frequency of abortion was higher in this group (100% *vs* 12.5%). In other studies, foetal death occurred in all cattle inoculated at day 70 of pregnancy with two high virulence isolates, Nc-Liv and Nc-Spain7, according to pathogenicity established in mice (Caspe *et al.,* 2012). In addition, the high virulent Nc-Spain7 isolate was compared with Nc-Spain8, which showed low-moderate virulence *in vitro* and in mouse models but a transplacental transmission rate higher than 50% (Regidor-Cerrillo *et al*., 2014). In contrast to observations made in the pregnant mice model, both isolates showed 100% of foetal mortality, although foetal death occurred earlier in Nc-Spain7 inoculated animals, which presented higher parasite burdens in placental and foetal tissues. In a recent study, IV inoculation of Nc-Spain7 at mid-gestation induced 50% of foetal death until 42 days post-infection (Almería *et al.,* 2016a) and 66.6% foetal death when gestation lasted until term (Vázquez *et al.,* submitted).

These studies highlighted the importance of the isolate in the outcome of infection in cattle and indicated that *N. caninum* isolates showing higher rate of invasion or tachyzoite yield *in vitro* as well as high virulence in mice are also more pathogenic in cattle than those moderate-low virulence isolates (Regidor-Cerrillo *et al.,* 2011; Dellarupe *et al.,* 2014a).

2.2.2.4 Molecular basis of the variability

N. caninum isolates have been isolated from diverse hosts and geographic regions, suggesting a high capacity of adaptation to different ecological niches, which could involve a high genetic diversity, as previously described in *T. gondii* (Grigg *et al.,* 2001). However, in contrast to *T. gondii*, the genetic basis of the biological variability in *N. caninum* remains unknown. The first studies of genetic variability among *N. caninum* isolates were based on the analysis of 18S rRNA, as well as the ITS-1 (Internal Transcribed Spacer-1) and the RAPD (Randomly Amplified Polymorphic DNA). The high conserved sequences of other analyzed markers indicated a lower genetic variability than in *T. gondii* (Beck *et al.,* 2009). Later, an extensive genetic diversity was demonstrated in *N. caninum* based on highly polymorphic microsatellite sequences (Regidor-Cerrillo *et al.,* 2006; Regidor-Cerrillo *et al.,* 2008; Pedraza-Díaz *et al.,* 2009; Basso *et al.,* 2009; Basso *et al.,* 2010; Regidor-Cerrillo *et al.,* 2013). The multilocus analyses have allowed to associate certain genetic profiles of each isolate to their country of origin (Regidor-Cerrillo *et al.,* 2013), although a link

between certain microsatellite markers and the virulence of *N. caninum* isolates have not been found.

In addition, the study of Single-Nucleotide Polymorphism (SNPs) allowed the definition of the population structure of *T. gondii*, with up to 15 haplogroups that include the classic type I, II and III strains. In fact, the type III strains present SNPs that affect the TgROP5, TgROP16 and TgROP18 genes, and determine their virulence (Taylor; *et al.,* 2006; Saeij *et al.,* 2006; Reese & Boothroyd, 2011; Behnke *et al.,* 2011). In *N. caninum*, the identification of SNPs has been carried out recently by sequencing different isolates. However, those analyses showed that all the genomes are broadly identical, suggesting that the current population of *N. caninum* is due to the expansion of a single clonal lineage that has been favoured by the vertical transmission of the parasite by asexual reproduction. However, mitochondrial DNA and DNA from the apicoplast presented important differences respect to the nuclear DNA, evidencing a process of sexual recombination in the definitive host that would explain the intra-specific variability of the different isolates of the parasite (Khan *et al.,* 2015).

Furthermore, protein expression profiles between high- and low-virulence isolates of *N. caninum* have been carried out, finding differences in the abundance and protein species involved in gliding motility, lytic cycle processes and oxidative stress (Regidor-Cerrillo *et al.,* 2012), which suggest the importance of variation on protein expression in the biological diversity of *N. caninum*.

Recent studies combining the "omics" techniques provide a global view of the elements involved in biological processes at the molecular level. The rise of the "omics" techniques began after the sequencing and publication of the genome of several important eukaryotic pathogens in the EuPathDB database (Eukaryotic Pathogen Database, www.eupathdb.org) (Aurrecoechea *et al.,* 2016). Specifically, the ToxoDB database (www.toxodb.org) groups freely and collaboratively the available "omics" information of *Eimeria*, *Hammondia*, *Toxoplasma*, *Neospora*, *Sarcocystis*, *Cystoisospora* and *Cyclospora* (Gajria *et al.,* 2007). High-throughput genome, transcriptome and proteome procedures are revealing molecular mechanisms involved in biological diversity and virulence of apicomplexan. Findings implicate variability in genetic background and regulation of gene and protein expression at the level of epigenetics, transcription, translation, and posttranslational modification. Advances in genomic, transcriptomic and proteomic studies of *N. caninum*, which have allowed to evidence some mechanisms responsible for the phenotypic traits of the different isolates and to identify the processes determining their virulence, are summarized below.

2.2.2.4.1 Genomic studies

Genomics comprises molecular characterization of complete genomes through the study of their content, organization, function of their genes and evolution of their genetic information. Up to date, the unique available genome sequence of *N. caninum* Nc-Liv isolate has 61 Mb, 14 chromosomes and more than 7,000 coding genes (Reid *et al.,* 2012) and has been compared to *T. gondii* genomes of TgME49 (Type II) and TgVEG (Type III).

More information is available for *T. gondii* than for *N. caninum*, and includes different isolates (ME49, GT1, VEG and RH). Both *T. gondii* and *N. caninum* genomes showed a high degree of synteny with a one-to-one correspondence between most of protein-coding genes (Reid *et al.,* 2012; Ramaprasad *et al.,* 2015), so that *T. gondii* has traditionally been used as a model for studies of *N. caninum*. However, there are also marked discrepancies on gene repertoire between both *T. gondii* and *N. caninum* genomes (Reid *et al.,* 2012). For example, several orthologous of virulence factors for *T. gondii* are pseudogenes such as ROP18 and GRA15 or were non-predicted from the Nc-Liv genome sequence such as GRA24. In addition, *new* putative genes associated to micronemes and rhoptries (SAG1-Related Sequence -SRS- gene family) such as Nc*S*RS67 were identified in *N. caninum* (Bezerra *et al.,* 2017).

On the other hand, the comparison of the genome of different apicomplexan parasites has defined the existence of families of genes that could be associated with evasion strategies of the immune system and other virulence factors (Reid, 2015), characterized for their high mutation rate, gene expansion and high polymorphism. In *T. gondii*, these families include the surface proteins SRS (Tomavo, 2001; Reid *et al.,* 2012) and the kinases present in the roptrias (ROPK) (Talevich & Kannan, 2013). In *N. caninum*, different protein-encoding genes have been sequenced and compared amongst isolates from different hosts and geographical areas (Beck *et al.,* 2009), resulting in poor gene polymorphism with likely limited phenotypic impact. However, clonal gene expansion, recently determined in *N. caninum*, may drive phenotypic variants by gene dosage (Adomako-Ankomah *et al.,* 2014). *N. caninum* contained predicted protein-coding genes involving SRS family, ROP and NTPase with attributed roles on virulence (Jung *et al.,* 2004; Adomako-Ankomah *et al.,* 2014; Pastor-Fernández *et al.,* 2016). Whether there is variation in the copy number within *N. caninum* remains to be determined.

Finally, as exposed before, the apicoplast, that has its own genetic material as well as its own gene expression machinery, seems to be essential for the parasite and, for this reason, its genome has been completely sequenced in *T. gondii*, *N. caninum* and some species of the genus *Plasmodium*.

2.2.2.4.2 Transcriptomic studies

Transcriptomics studies gene expression at one specific stage of development of a cell by analysing transcribed RNA. This information supposes a link between genomic and proteomic findings, although it does not consider regulation occurred between transcription and translation.

Transcriptome analyses were firstly performed in *T. gondii* tachyzoites using SAGE (Serial Analysis Gene Expression), ESTs (expressed sequence tag), microarrays, MPSS (Massively Parallel Signature Sequencing) and TSSs (Transcription Start Site) techniques (Radke *et al.,* 2005; Wastling *et al.,* 2009; Yamagishi *et al.,* 2010; Hassan *et al.,* 2012). Mechanisms of host cell manipulation after infection have been investigated by transcriptomics. For example, it was discovered that TgROP16, TgGRA7 and TgGRA15 proteins may alter certain transcription routes after infection by different mechanisms (Yamamoto *et al.,* 2009; Ong *et al.,* 2010; Rosowski *et al.,* 2011; Jensen *et al.,* 2013; Bougdour *et al.,* 2014). Interestingly, many of these factors presented important polymorphisms between different *T. gondii* isolates, conditioning certain characteristics of the

infection (Taylor *et al.,* 2006; Saeij *et al.,* 2006; Reese & Boothroyd, 2011; Behnke *et al.,* 2011). On the other hand, an increase in the transcription of micro-RNAs and the c-Myc gene in infected host cells was described and related to regulation of transcription, progression of cell cycle and apoptosis (Zeiner & Boothroyd, 2010; Franco *et al.,* 2014). Transcriptomic studies also revealed that infection by *T. gondii* increased the expression of immune response genes and that expression varied according to the implicated isolate (Gail *et al.,* 2001; Chaussabel *et al.,* 2003; Okomo-Adhiambo *et al.,* 2006; Skariah *et al.,* 2010; Jia *et al.,* 2013; Tanaka *et al.,* 2013; Pittman *et al.,* 2014).

Nowadays, there are few studies of the transcriptome of *N. caninum*. The comparison between *N. caninum* and *T. gondii* tachyzoite transcriptome showed an expansion of the genes encoding the SRS surface proteins, as well as a lower or nonexistent transcription of virulence factors involved in mechanisms of pathogenicity of *T. gondii* (NcROP18, NcROP16, NcROP5, NcSUB2) (Reid *et al.,* 2012). Species-specific gene repertoire may explain the biological differences of host restriction, transmission strategies, zoonotic potential and the limited pathogenicity of *N. caninum* in mice (Reid *et al.,* 2012). In addition, it has been demonstrated that *N. caninum* cannot increase the transcription of micro-RNAs and the c-Myc gene, as observed in *T. gondii* (Zeiner & Boothroyd, 2010; Franco *et al.,* 2014).

On the other hand, controlled changes in *N. caninum* transcriptome are expected during the lytic cycle in host cells, during differentiation in specific stages, and during its exposition to different environment and immune responses in the host. The Apetala2 (ApiAP2) family is the major group of transcription factors in apicomplexan controlling parasite cell cycle progression, stage transformation and virulence, and 54 putative NcApiAP2s may be differentially expressed or regulated among *N. caninum* isolates determining phenotypic variants similarly to described in *T. gondii* (Croke *et al.,* 2014; Melo *et al.,* 2015). In addition, post-transcriptional regulation for apiAP2 factors has been suggested (Ramaprasad *et al.,* 2015). Moreover, an extensive repertoire of histone modification machinery is present in apicomplexan, including *N. caninum*, although its "histone code" has not been explored (Bougdour *et al.,* 2010; Dixon *et al.,* 2010; Nardille *et al.,* 2013). Recently, a putative set of natural long antisense transcripts (lancRNA) and long intergenic non-coding RNAs (lincRNAs) has been identified in *N. caninum* (Ramaprasad *et al.,* 2015). NcRNAs can interfere in post-transcriptional regulation of gene expression through RNA splicing, transport or nuclear retention of the corresponding sense RNA transcripts, mRNA stability and modulation of translation in eukaryotes (Villegas & Zhapiropoulos, 2015).

In the last years, SALUVET group has carried out transcriptomic studies that provide information on the mechanisms used for two different virulence isolates of *N. caninum* to interact with the host cell. In a first work, RNA-Seq was used to investigate differences in transcriptome between Nc-Spain7, a high-virulence isolate, and Nc-Spain1H, a low-virulence isolate, when infecting MARC-145 culture cells. Transcriptomes from both isolates showed marked variations throughout the lytic cycle but were inconsistent with proteome results carried out in the same conditions. However, a pre-bradyzoite status of the low virulence isolate Nc-Spain1H was identified (Horcajo *et al.,* 2018). In a second work, differential expression of *N. caninum* genes involved in host cell attachment and invasion (SAG-related and microneme proteins), glideosome, rhoptries, metabolic processes, cell cycle and stress response between both isolates was found in F3 infected with both isolates, evidencing their intra-specific variability (Horcajo *et al.,* 2017).

In the last work, it was demonstrated that Nc-Spain7 and Nc-Spain1H infection modulate bovine macrophage host signaling pathways to escape cellular defenses by repression of apoptosis and lysosome degradation. In addition, differences between isolates were compared, finding a greater modulation by Nc-Spain1H infection (García-Sánchez *et al.,* submitted). Another recent work compared the tachyzoite transcriptomes of Nc-Liv (virulent) and Nc-Nowra (avirulent) and identified 3130 SNPs and 6123 indels between both isolates (Calarco *et al.,* 2018). Nine markers were Sanger sequenced for both strains and for other additional eight strains, identifying a genetic population structure comprised of two major clades with no geographical segregation. The variants were predominantly located in loci associated with protein binding, protein-protein interactions, transcription, and translation. Furthermore, 468 nonsynonymous SNPs identified within protein-coding genes were associated with protein kinase activity, protein binding, protein phosphorylation, and proteolysis. This work may implicate these processes and the specific proteins involved as novel effectors of *N. caninum* tachyzoite virulence.

2.2.2.4.3 Proteomic studies

Proteomics comprises the global analysis of the proteins expressed at a specific time-point, and have emerged as very useful tools for the study of apicomplexan parasites, establishing their protein repertoire and understanding the biological processes involved in stage transformation and host-cell interactions (Weiss *et al.,* 2009; Wastling *et al.,* 2009; Marugán-Hernández *et al.,*2010; Bautista *et al.,* 2014). It is assumed that *N. caninum* proteome is reflect of transcriptome, although observed discrepancies between the transcriptome and proteome in apicomplexan suggest additional translational control on their proteome (Xia *et al.,*2008; Wastling *et al.,* 2009).

First proteomic approaches used two-dimensional electrophoresis (2-DE) to define the protein composition of *N. caninum* tachyzoites (Lee *et al.,* 2003; Lee *et al.,* 2004), and to compare tachyzoite proteome between different *N. caninum* isolates, showing similar proteomes with variation in a small number of isolate-specific proteins (Lee *et al.,* 2005; Shin *et al.,* 2005a; Shin *et al*., 2005b). The proteomes of *N. caninum* and *T. gondii* were also compared by 2-DE (Zhang *et al.,* 2011). The development of Two Dimensional-Difference Gel Electrophoresis technique (2D-DIGE) allowed to quantify the differences in abundance of those proteins differentially expressed between two different extracts. Comparison of the protein expression profiles of tachyzoite of two high (Nc-Liv and Nc-Spain7) and one low virulence isolate (Nc-Spain 1H) demonstrated the increased abundance of proteins and variation of the protein species involved in the gliding motility (e.g., ACT1 and MLC1), lytic cycle processes (e.g., NcROP40 and NcMIC1), and oxidative stress responses (e.g., G6PDH) of virulent isolates (Regidor-Cerrillo *et al.,* 2012). Remarkably, a high number of protein species with variation in abundance were identified as the same protein, which suggest additional and different regulation by post-translational modification, protein turn-over and degradation among isolates in *N. caninum*. A later study comparing Nc-Spain7 and Nc-Spain1H showed different proteomes throughout the lytic cycle and a pre-bradyzoite status of the low virulence isolate Nc-Spain1H was identified (Horcajo *et al.,* 2018). Thus, these studies suggest that variations in the levels of protein expression and modulation could be the predominant force driving the biological diversity of *N. caninum*.

On the other hand, the study of the antigenic diversity among *N. caninum* isolates revealed a low intraspecific variability, since the analysis of different isolates by WB showed similar profiles (Schock *et al.,* 2001; Miller *et al.,* 2002; Shin *et al.,* 2005a; Shin *et al.,* 2005b; Lee *et al.,* 2005). A recent work suggested that there may be a different host immune response against different isolates (Regidor-Cerrillo *et al.,* 2015). In this study, immunomes from two virulent isolates (Nc-Liv, Nc-Spain7) and a low virulent isolate (Nc-Spain1H) were compared, confronting the protein extracts with sera from mice infected with each isolate. The differences found were dependent on the serum and not on the extract analyzed, suggesting that the differences in the immunome are due to the immune response induced by each isolate.

Finally, proteomic approaches have also been used to compare the proteome of the tachyzoite and bradyzoite phases (Marugán-Hernández *et al.,* 2010), to identify specific proteins of secretion organelles (Marugán-Hernández *et al.,* 2011a; Sohn *et al.,* 2011), as well as antigens that stimulate bovine T cells CD4+ (Rocchi *et al.,* 2011) and secreted proteins by artificially inducing the release of intracellular calcium deposits (Pollo-Oliveira *et al.,* 2013).

CHAPTER III

JUSTIFICATION AND OBJECTIVES

JUSTIFICACIÓN Y OBJETIVOS

Neospora caninum is an apicomplexan cyst-forming protozoan parasite that is considered as one of the main causes of abortion in cattle worldwide, including Spain. Important economic losses associated with the disease have been quantified, mainly as a consequence of the reproductive failure (Dubey & Schares, 2011). Transplacental transmission is the main route of transmission in cattle leading to abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Innes *et al.*, 2002), playing the bovine placenta a key role in the pathogenesis of this disease.

The direct damage produced by the multiplication of the parasite in placental and foetal tissues has been proposed as one of the possible causes of foetal death observed during *N. caninum* natural infections. Invasion of the placenta, proliferation and dissemination to the foetus seem to be crucial events in the pathogenesis of bovine neosporosis (Hemphill *et al*., 2006). However, the mechanisms by which *N. caninum* infects the placenta and reaches the foetus are poorly studied (Robbins *et al*., 2012). Importantly, in addition to be the main barrier for parasite transmission, the placenta is considered an immune regulatory organ, modulating foetal and maternal immune responses. It has been shown that the multiplication of the parasite in the placenta (and in other maternal tissues/organs) may alter the immunological balance at the maternal-foetal interface, suggesting immuno-mediated pathogenesis as a possible cause of abortion (Entrican, 2002; Quinn *et al*., 2002b; Innes *et al*., 2007; Bartley *et al*., 2012; Bartley *et al*., 2013). Trophoblast and caruncular cell layers constitute the maternal-foetal interface. These cells are able to recognize pathogens and secrete cytokines and chemokines, recruiting immune cells in the damaged area (Montes *et al*., 1995; Steinborn *et al.,* 1998a; Steinborn *et al*., 1998b), and, thus, participacing in the initiation of innate immune responses at the placental level as well as in the development of an adaptative immune response. However, those cytokines and chemokines could contribute to abortion or facilitate parasite passage across the placenta and congenital transmission (Entrican, 2002; Innes, 2007). On the other hand, modified expression of adhesion receptors in the placenta could determine parasite passage. Selective expression of these cytokines and adhesion molecules, as a consequence of placental infection, may also reflect virulence mechanisms.

Parasite-host interactions are also determined by the parasite intra-specific variability, which also supose an essential factor on the outcome of infection. Previous findings demonstrated that *N. caninum* isolates of bovine or canine origin show a large biological diversity, although they are genetically similar (Regidor-Cerrillo *et al*., 2011). Differences among several *N. caninum* isolates *in vitro* were correlated with differences observed in animal models and in the cytokine profiles induced during the infection (Regidor-Cerrillo *et al.,* 2011; Dellarrupe *et al*., 2014a; Dellarupe *et al*., 2014b). Specifically, pregnant heifers inoculated at day 70 of gestation with the low-virulence isolate Nc-Spain1H spared the foetus (Rojo-Montejo *et al*., 2009b), whereas foetal death occurred in all inoculated cattle with the highly virulent isolate Nc-Spain7 (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014).

In vitro models suppose an alternative to *in vivo* experiments, having numerous applications, and, concretely, *in vitro* culture systems represent powerful tools for the study of apicomplexan parasites (Muller &Hemphill, 2012). In addition to the reduction of experimental animals, they facilitate the initial approach to certain mechanisms involved in complex processes obtaining reproducible results. Despite the great utility of *in vitro* models, the use of animal models is

essential for the study of several aspects of the disease. Although murine and ovine models have been utilized for the study of bovine neosporosis, those models did not reproduce faithfully the disease due to species-specific physiological differences in the structure of the placenta and in the immunological mechanisms developed against pathogen infection. Moreover, cattle is the most relevant and economically important target host, so that investigations related to neosporosis should be performed in the target animal species, despite to economic constraints and space-limitations.

In this scenario, the **general aim of the present Doctoral Thesis was to investigate the interactions between two *N. caninum* isolates with marked differences in virulence and the cells that conform the maternal-foetal interface in the bovine placenta both *in vivo* and *in vitro***. The final purpose is to bring light to the role of the bovine placenta in the pathogenesis of neosporosis, and the phenotypic factors that enable some isolates to be more effectively transmitted and cause fetal death than others.

The Spanish isolates Nc-Spain7 and Nc-Spain1H were used in all the experiments. The high-virulence isolate Nc-Spain7 was obtained from an asymptomatic calf (Regidor-Cerrillo *et al*., 2008) and has been thoroughly characterized in both *in vitro* and *in vivo* (murine, ovine and bovine) models (Regidor-Cerrillo *et al.*, 2010; Regidor-Cerrillo *et al*., 2011; Caspe *et al.*, 2012; Collantes-Fernández *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014; Dellarupe *et al.*, 2014a; Dellarupe *et al*., 2014b; Arranz-Solís *et al*., 2016; Almería *et al*. 2016a; Almería *et al*., 2016b; García-Sánchez *et al*., 2019; Vázquez *et al*., submitted). The low-virulence isolate Nc-Spain1H was obtained from the brain of a clinically healthy calf from a Spanish herd with high intra-herd *N. caninum* seroprevalence (Rojo-Montejo *et al*., 2009a). Nc-Spain1H was also characterized *in vitro* (Rojo-Montejo *et al*., 2009a; Regidor-Cerrillo *et al*., 2010; García-Sánchez *et al*., 2019) and *in vivo* (Rojo-Montejo *et al*., 2009a; Rojo-Montejo *et al*., 2009b). The potential of this isolate as a live vaccine candidate was also investigated due to its attenuated nature (Rojo-Montejo *et al.*, 2013). Previous findings showed the biological diversity between both isolates (Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2010; Regidor-Cerrillo *et al*., 2011; Collantes-Fernández *et al.*, 2012; Regidor-Cerrillo *et al*., 2014; García-Sánchez *et al*., 2019), despite of apparently poor genetic variability (Regidor-Cerrillo *et al*., 2013). Concretely, differences in the lytic cycle of Nc-Spain7 and Nc-Spain1H were found *in vitro* (Regidor-Cerrillo *et al*., 2011; Dellarupe *et al.*, 2014b; García-Sánchez *et al*., 2019), mainly in the proliferation ability, which was clearly enhanced in Nc-Spain7. Moreover, differences found *in vitro* are correlated with differences observed in virulence and vertical transmission in animal models. Specifically, higher parasite burden and more severe lesions were observed in a pregnant mice model infected with Nc-Spain7 together with high rates of neonatal mortality and vertical transmission (Regidor-Cerrillo *et al*., 2011; Dellarupe *et al*., 2014a), whereas 0.5% and 5% of neonatal mortality and foetal transmission were detected in mice infected with Nc-Spain1H (Rojo-Montejo *et al*., 2009a). In pregnant bovine models, 100% of foetal mortality and vertical transmission was observed after Nc-Spain7 infection at early gestation (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014), and at least 50% of foetal mortality at mid-gestation (Almería *et al*., 2016a; Vázquez *et al*., submitted). However, Nc-Spain1H infection spared the foetus at early gestation (Rojo-Montejo *et al*., 2009b).

In vitro cultures of two bovine placental target cells that conform the maternal-foetal interface (trophoblast and caruncular cells) have been used in the present Doctoral Thesis in order to characterize the parasite-host interactions. Bovine caruncular epithelial cells, named BCEC-1, and

bovine trophoblast cells, named F3, kindly provided by Prof. Pfarrer from the Anatomy Institute of the University of Veterinary Medicine of Hannover, were obtained by trypsin digestion of caruncular and cotyledonary tissues after manual separation of bovine placentomes from 4 and 5 months pregnant cows, respectively. Both established cultures were obtained through spontaneous immortalization, which resulted positive in order to avoid the loss of specific cell properties derived from the immortalization of cells by techniques such as gamma rays or different viral systems, as was observed in human trophoblast (Khoo *et al*., 1998). In addition, both the origin and the presence of specific properties from the original tissues were assessed by different studies (Bridger *et al*., 2007a; Bridger *et al*., 2007b; Hambruch *et al*., 2010; Waterkotte *et al*., 2011), concluding that these cell cultures would represent good tools for the study of regulatory mechanisms of placental growth and function.

In the present Doctoral Thesis, the following specific objectives were pursued:

- **Objective 1:** Characterization of parasite interaction between high- and low-virulence isolates of *N. caninum* and bovine placental target cells *in vitro*.

The aim of this objective was to bring light into the events that take place in the placenta after infection with different virulence isolates. The different phases of the lytic cycle in caruncular and trophoblast cells as well as the modulation of the immune responses elicited by the infection in these cells were investigated. The results will allow us to find differences between isolates in the maternal-foetal interface of the bovine placenta and to discern whether isolates of different virulence induce a different modulation of the placental cells during the early phases of infection. Moreover, comparisons between two isolates with marked differences in virulence are useful to unveil critical phases of the lytic cycle that may determine the differences observed among them, and virulence mechanisms using by *N. caninum* to avoid immune responses and to cross the placental barrier.

  - Sub-objective 1.1: Characterization of the lytic cycle of high- and low-virulence isolates of *N. caninum* in bovine placental target cells *in vitro*.

In this sub-objective investigation of tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells was carried out with the aim of evaluating critical factors involved in placental pathogenesis.

  - Sub-objective 1.2: *In vitro* interaction between *N. caninum* and the placental target cells from an immunological level.

In this study, expression profiles of toll-like receptor-2 (TLR-2), Th1 and Th2 cytokines (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17A, IFN-γ, TGF-β1 and TNF-α), and endothelial adhesion molecules (ICAM-1 and VCAM-1) were evaluated in bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells after the infection with high- (Nc-Spain7) and low- (Nc-Spain1H) virulence isolates of *N. caninum.* Results from this sub-objective will help to establish the role of placental cells in the regulation of the innate immune responses at the placental level and their reprogramming by *N. caninum* infection with isolates of variable virulence.

- **Objective 2:** Investigation of the early infection by high- and low-virulence isolates of *N. caninum* in pregnant cattle at mid gestation .

In vitro findings were also tested in experimental infections in cattle. Comparative analysis of the high- and low-virulence isolates will help to identify the role of immune responses at the maternal-foetal interface in the clinical outcome or in the control of parasite transmission and proliferation in placental and foetal tissues. Special emphasis was given to investigate the consequences of parasite infection in the placenta *in vivo* based upon immune cell population and expression profiles of immune elements, and other key markers of interest related to innate immune response and pathogenesis.

  - Sub-objective 2.1: Early *N. caninum* infection dynamics in pregnant heifers after inoculation at mid-gestation with high- and low-virulence isolates.

The aim of this sub-objective was to investigate how the differences between high- (Nc-Spain7) and low- (Nc-Spain1H) virulence isolates of *N. caninum* influence the clinical outcome, parasite distribution and burden, lesion development in placental and foetal tissues, and the specific antibody response during early infection in pregnant heifers inoculated at mid-gestation. The results from this experimental model will clarify some of the key events involved in the pathogenesis of bovine neosporosis, providing new insight into the host-pathogen interactions in the placenta and the foetus and validating results obtained *in vitro*.

  - Sub-objective 2.2: Placental immune response and extracellular matrix organization during the early stages of *N. caninum* infection in pregnant heifers inoculated with high- and low-virulence isolates at mid-gestation.

This sub-objective included the study of the variations in the mRNA and protein expression of elements of the innate and adaptative immune responses as pattern recognition receptors (PRRs) (TLR-2, -3, -8 and -9 and NOD-2), pro-inflammatory (IL-1β, IL-6, IL-8, IL-12p40, IL-17A, IFN-γ and TNF-α) and anti-inflammatory/regulatory cytokines (IL-4, IL-10 and TGF-β1), iNOS, chemokines (CCL2, 4 and 5) and endothelial adhesion molecule genes (ICAM-1 and VCAM-1) triggered locally in the bovine placenta at two early time-points (10 and 20 dpi) after inoculation with Nc-Spain7 and Nc-Spain1H isolate. The inflammatory infiltrate (T and B lymphocytes and phagocytic cells) was also studied. Finally, the regulation of the extracellular matrix was investigated by analysing the mRNA and protein expression levels of its regulators (MMP-2, -13, -14, TIMP-1 and -2) and the protein expression of its components (fibronectin, vimentin and collagen type IV). These studies will allow to identify association of the infection with subversion or induction of protective responses and investigate critical immune elements involved in placental pathogenesis as well as changes in the ECM of the placenta. These results will help to confirm and validate those obtained in the *in vitro* models.

CHAPTER IV

RESULTS (PUBLICATIONS)

RESULTADOS (PUBLICACIONES)

LIST OF PUBLICATIONS

Objective 1: Characterization of parasite interaction between high- and low-virulence isolates of *N. caninum* and bovine placental target cells *in vitro*.

Sub-objective 1.1: Characterization of the lytic cycle of high- and low-virulence isolates of *N. caninum* in bovine placental target cells *in vitro*.

Jiménez-Pelayo L, García-Sánchez M, Regidor-Cerrillo J, Horcajo P, Collantes-Fernández E, Gómez-Bautista M, Hambruch N, Pfarrer C, Ortega-Mora LM. Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with high and low virulence isolates of *Neospora caninum*. Published in *Parasites & Vectors*. (17th October 2017) 10:463. DOI: 10.1186/s13071-017-2409-9.

Sub-objective 1.2: *In vitro* interaction between *N. caninum* and the placental target cells from an immunological level.

Jiménez-Pelayo L, García-Sánchez M, Regidor-Cerrillo J, Horcajo P, Collantes-Fernández E, Gómez-Bautista M, Hambruch N, Pfarrer C, Ortega-Mora LM. Immune response profile of caruncular and trophoblast cell lines infected by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *Neospora caninum*. Published in *Parasites & Vectors*. (8th May 2019) 12:218. DOI: 10.1186/s13071-019-3466-z.

Objective 2: Investigation of the early infection by high- and low-virulence isolates of *N. caninum* in pregnant cattle at mid-gestation.

Sub-objective 2.1: Early *N. caninum* infection dynamics in pregnant heifers after inoculation at mid-gestation with high- and low-virulence isolates.

Jiménez-Pelayo L, García-Sánchez M, Vázquez P, Regidor-Cerrillo J, Horcajo P, Collantes-Fernández E, Blanco-Murcia J, Gutiérrez-Expósito D, Román-Trufero A, Benavides J, Ortega-Mora LM. Early *Neospora caninum* infection dynamics in cattle after inoculation at mid gestation with high (Nc-Spain7)- or low (Nc-Spain1H)-virulence isolates. Submitted to *Veterinary Research* (18th June 2019).

Sub-objective 2.2: Placental immune response and extracellular matrix organization during the early stages of *N. caninum* infection in pregnant heifers inoculated with high- and low-virulence isolates at mid-gestation.

Jiménez-Pelayo L, García-Sánchez P, Collantes-Fernández E, Regidor-Cerrillo J, Horcajo P, Gutiérrez-Expósito D, Espinosa J, Benavides J, Osoro K, Pfarrer C, Ortega-Mora LM. Differential immune responses and extracellular matrix organization are observed in the bovine placenta during early infection at mid-gestation with high- and low-virulence *Neospora caninum* isolates. *Manuscript in preparation.*

Objetivo 1

Caracterización de la interacción parásito-hospedador entre aislados de alta y baja virulencia de *N. caninum* y las células diana de la placenta bovina *in vitro*

Neospora caninum supone una de las principales causas de aborto en el ganado bovino en todo el mundo en parte gracias a su gran capacidad para cruzar la barrera placentaria. De esta forma, el daño causado por el parásito en su paso por la placenta se ha indicado como uno de los factores clave en la patogénesis de la neosporosis. Además, la infección con *N. caninum* supone un desafío para el sistema inmunitario de las vacas gestantes. De hecho, los desequilibrios en el balance Th1/Th2 en la placenta durante la gestación como consecuencia de la infección por *N. caninum* también se han propuesto como una posible causa del aborto. Los trofoblastos, células epiteliales fetales, componen la interfaz materno-fetal en el placentoma bovino junto con las células carunculares maternas. Ambos tipos celulares son componentes claves de dicha interfaz, jugando un papel fundamental en la funcionalidad de la placenta. De hecho, tanto las células del cotiledón como las de la carúncula son capaces de reconocer a patógenos, *N. caninum* entre ellos, e inducir una respuesta inmunitaria frente a ellos. En este objetivo se estudió el ciclo celular de dos aislados de *N. caninum* de alta y baja virulencia *in vitro*, en cultivos establecidos de células del trofoblasto y la carúncula bovina, así como se caracterizó la respuesta inmunitaria inducida por la infección en ambos tipos celulares en un momento temprano y otro más tardío de la infección (4 y 24 horas post-infección). En concreto, la adhesión, la invasión, la proliferación y la egresión de los taquizoítos de los aislados Nc-Spain7 y Nc-Spain1H fueron evaluadas mediante ensayos de inmunofluorescencia en placa y PCR cuantitativa, mientras que la respuesta inmunitaria se valoró mediante PCR cuantitativa del ARNm del receptor tipo toll 2 (TLR-2), citoquinas Th1 y Th2 (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17, IFN-γ, TGF-β1, TNF-α) y moléculas de adhesión endotelial (ICAM-1 y VCAM-1), y mediante la medición de los niveles de proteína de la IL-6, la IL-8 y el TNF- α por ELISA.

N. caninum invadió y se multiplicó en ambas líneas celulares, aunque las mayores tasas de invasión (pInvR), infección (cInfR) y proliferación (TY_{58H}) se encontraron en trofoblastos infectados con el aislado más virulento Nc-Spain7, que sugirió que los trofoblastos pueden ser el nicho preferido de replicación del parásito. La multiplicación en ambas células fue exponencial entre las 22 hpi y las 58 hpi, excepto para el aislado menos virulento, Nc-Spain1H, que mostró no ajustarse a un crecimiento exponencial ni lineal en las células de la carúncula. En dichas células, una egresión temprana de ambos aislados se observó a partir de las 22 hpi, hecho interesante que no había sido observado anteriormente y que demuestra el interés de este tipo celular y sugiere un papel como barrera en la placenta bovina. Observando las diferencias entre aislados, el aislado más virulento mostró mejor capacidad de penetración en las células del trofoblasto, así como mayor tasa de infección y multiplicación que el aislado menos virulento Nc-Spain1H. Sin embargo, las diferencias entre los aislados fueron mucho menos marcadas en el caso de las células de la carúncula sugiriendo un papel importante de los trofoblastos en la patogénesis de la infección.

Por otro lado, la valoración de la respuesta inmunitaria mostró un patrón similar de expresión de ARNm en ambas líneas celulares, encontrándose una rápida respuesta pro-inflamatoria, con el aumento de citoquinas como el TNF-α y la IL-8 en las células infectadas y una disminución de citoquinas anti-inflamatorias/regulatorias como la IL-6 y el TGF-β1. Los niveles proteicos de IL-6, IL-8 y TGF-β1 en el sobrenadante confirmaron los resultados de los niveles de expresión de ARNm. Se observaron además pequeñas diferencias entre los aislados, con una mayor expresión de TLR-2 y TNF-α en ambas líneas celulares infectadas con el aislado Nc-Spain1H, que podrían ayudar al control del aislado menos virulento por parte del sistema inmunitario, lo que explicaría, al menos parcialmente, las diferencias encontradas entre los dos aislados.

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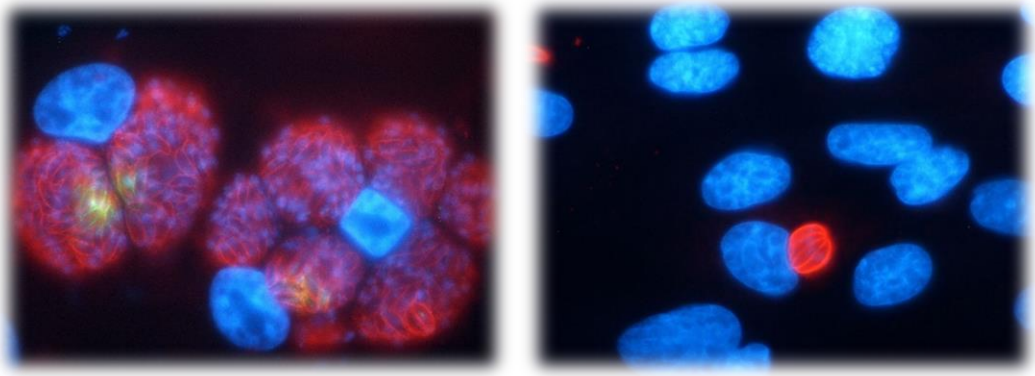
Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with high and low virulence isolates of *Neospora caninum*

Laura Jiménez-Pelayo[1†], Marta García-Sánchez[1†], Javier Regidor-Cerrillo[1], Pilar Horcajo[1], Esther Collantes-Fernández[1], Mercedes Gómez-Bautista[1], Nina Hambruch[2], Christiane Pfarrer[2] and Luis Miguel Ortega-Mora[1*]

[1]SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[2]Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

[†]Authors contributed equally



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Abstract

Background: *Neospora caninum*, one of the main causes of abortion in cattle, is very effective at crossing the placental barrier and placental damage is crucial in the pathogenesis of abortion. Bovine trophoblast and caruncular cell layers are key cellular components in the maternal-foetal interface in placentomes, playing a fundamental role in placental functionality.

Methods: We studied tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence *N. caninum* isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells. The parasite invasion rate (pInvR) and the cell infection rate (cInfR) were determined by immunostaining plaque assay at different time points and multiplicities of infection (MOIs), respectively. In addition, tachyzoite growth kinetics were investigated using real-time PCR (qPCR) analysis and immunostaining plaque assay at different times.

Results: *Neospora caninum* invaded and proliferated in both cell lines. The pInvR was higher in F3 compared to BCEC-1 cells for the Nc-Spain7 isolate ($P < 0.05$), and higher for the Nc-Spain7 than the Nc-Spain1H in F3 cells ($P < 0.01$). The cInfR was also higher in F3 cells than in BCEC-1 cells for both isolates ($P < 0.0001$), and the cInfR for the Nc-Spain7 isolate was higher than for the Nc-Spain1H isolate in both cell lines ($P < 0.05$). Tachyzoite growth kinetics showed tachyzoite exponential growth until egress at 58 hours post-infection (hpi) for both isolates in F3, whereas Nc-Spain1H showed a non-exponential growth pattern in BCEC-1. Asynchronous egress of both isolates was observed from 22 hpi onwards in BCEC-1. In addition, the tachyzoite yield (TY_{58h}) was higher in F3 than in BCEC-1 infected by both isolates ($P < 0.0001$), highlighting better replication abilities of both parasites in F3. Nc-Spain7 showed shorter doubling times and higher TY_{58h} compared to Nc-Spain1H in F3 cells; adhesion, invasion and proliferation mechanisms were very similar for both isolates in BCEC-1.

Conclusions: Our results indicate a highly similar behavior of high- and low-virulence isolates in their interactions with maternal caruncular cells and suggest an important role of foetal trophoblasts in the pathogenesis of *N. caninum* infection.

Keywords: *Neospora caninum*, Cattle, Isolates, Virulence, Placenta, Trophoblast, Caruncle, Adhesion, Invasion, Proliferation

1. Background

Neospora caninum is an apicomplexan protozoan parasite, phylogenetically related to *Toxoplasma gondii*. This parasite is considered a major cause of reproductive failure in cattle worldwide (Innes *et al*., 2005; Dubey *et al* 2006; Dubey *et al*., 2007), resulting in great economic losses (Reichel *et al*., 2013). Infection in cattle may occur through horizontal transmission, when cattle ingest sporulated oocysts shed by a canid definitive host, or by endogenous congenital transmission, from a persistently infected dam to a foetus (Williams *et al*., 2009). Oral infection or recrudescence in a pregnant cow can result in abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Dubey *et al*., 2007).

Neospora caninum is one of the most efficiently transplacentally-transmitted organisms in cattle (Williams *et al*., 2009). During natural infections, invasion of the placenta, proliferation and dissemination to the foetus are crucial events in the pathogenesis of bovine neosporosis and are related to the interactions of tachyzoites with host cells and its relationship with the local immune response at the maternal-foetal interface (Hemphill *et al*., 2006). *In vivo* studies demonstrated that *N. caninum* is able to infect the maternal caruncular septum before crossing to the foetal placental villus (Gibney *et al*., 2008; Benavides *et al*., 2014). Despite

the fact that the placenta is directly involved in the pathogenesis of the disease (Entrincan, 2002; Innes, 2007), the mechanisms by which *N. caninum* infects the placenta and reaches the fetus are poorly understood (Robbins *et al*., 2012). One reason could be the placental diversity (Leiser & Kaufman, 1994), which makes an extrapolation of findings from one species to the other difficult. To date, only one limited *in vitro* study investigating the potential involvement of bovine trophoblast in *N. caninum* infection has been published (Machado *et al*., 2007). In addition, no information is available regarding *in vitro* infection in bovine caruncular epithelial cells and the role of placental cell layers in vertical transmission.

In addition, a key question in bovine neosporosis is the influence of the parasite intra-specific variability on the outcome of infection. The lytic cycle of *N. caninum* and other apicomplexan parasites comprises the processes of invasion, adaptation to intracellular conditions, proliferation, and egress from host cells (Hemphill *et al*., 2006; Plattner & Soldati-Favre, 2008; Santos *et al*., 2009). This sequence of events is required for parasite survival and propagation in the course of animal infection. Our previous findings demonstrated that *N. caninum* isolates of bovine or canine origin show a large biological diversity, despite being genetically similar (Regidor-Cerrillo *et al*., 2011). Moreover, differences found in the events of the lytic cycle among several *N. caninum* isolates *in vitro* are correlated with differences observed in virulence and vertical transmission in animal models (Regidor-Cerrillo *et al*., 2011; Dellarupe *et al*., 2014b). Specifically, pregnant heifers inoculated at day 70 of gestation with the low-virulence isolate Nc-Spain1H spared the foetus (Rojo-Montejo *et al*., 2009b), whereas foetal death occurred in all inoculated cattle with the highly virulent isolate Nc-Spain7 (Caspe *et al* 2012; Regidor-Cerrillo *et al*., 2014).

There is no information concerning the kinetics of events in the placenta that lead to an understanding of how the parasite actually reaches the foetal tissues. The influence of biological variability of the isolate on placental damage is also poorly understood. The cow possesses a cotyledonary (Strahl, 1906) and synepitheliochorial placenta (Wooding, 1992), where foetal cotyledons interdigitate with maternal caruncles to form placentomes (Bridger *et al*., 2007a; Bridger *et al* 2007b; Zeiler *et al*., 2007). The trophoblast (epithelial surface of the foetal cotyledons) consists of uninucleated and binucleated cells. Binucleated cells are responsible for a "restricted" trophoblast invasion (Pfarrer *et al*., 2003), playing an important role in embryo implantation and successful pregnancy outcomes. Caruncular epithelial cells form a polarized barrier that the parasite encounters before reaching and multiplying in foetal tissues. Hence, the aim of this study was to investigate the interaction of two isolates of *N. caninum* with maternal and foetal bovine target cells. Here, we studied tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells. Since BCEC-1 and F3 cells conserve some of the properties from their tissues of origin (Bridger *et al*., 2007b; Waterkotte *et al*., 2011; Hambruch *et al*., 2010), they are a useful tool to evaluate critical factors involved in placental pathogenesis, such as the mechanisms used by *N. caninum* to cross the placental barriers.

2. Methods

2.1 Parasites and cell cultures

Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy, congenitally infected calves (Regidor-Cerrillo *et al*., 2008; Rojo-Montejo *et al*., 2009a) and extensively characterized using *in vitro*, murine and bovine models (Rojo-Montejo *et al*., 2009a; Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2010; Regidor-Cerrillo *et al*., 2011; Regidor-Cerrillo *et al*., 2013; Regidor-Cerrillo *et al*., 2014). Tachyzoites were routinely maintained in a monolayer culture of the MARC-145 monkey kidney cell line as described previously (Regidor-Cerrillo *et al*., 2011). The *N. caninum* isolates used in this study were subjected to a limited number of culture passages (from 8 to 15) to ensure the maintenance of their *in vivo* biological behaviour and avoid their adaptation to the host cells (Pérez-Zaballos *et al*., 2005).

A bovine trophoblast cell line F3 (Hambruch *et al*., 2010) and a bovine caruncular cell line BCEC-1 (Bridger *et al*., 2007b) were isolated from two BVD-free, pregnant cattle (*Bos taurus*) with an estimated gestational age of 5 and 4 months, respectively. Cells were grown as indicated by Hambruch *et al*. (2010). Briefly, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 containing 10% foetal calf serum (FCS) checked for the absence of specific IgG against *N. caninum* by IFAT, 100 IU/ml Penicillin, 100 mg/ml Streptomycin

and 2 mM Glutamine. All experiments were carried out with cells below passage 27, when both cell lines maintained their morphological and functional features (Bridger *et al*., 2007b; Hambruch *et al*., 2010; Waterkotte *et al*., 2011).

Tachyzoites used for *in vitro* assays were recovered from 2.5–3 day growth cultures of MARC-145, when the majority of the parasites were still intracellular, and purified using Disposable PD-10 Desalting Columns (G.E. Healthcare, Buckinghamshire, UK) as previously described (Regidor-Cerrillo *et al*., 2011). Tachyzoite viability was checked by trypan blue exclusion. F3 and BCEC-1 cell monolayers were inoculated within 1 hour of tachyzoite collection from flasks. All *in vitro* experiments in F3 and BCEC-1 cell lines were assayed in quadruplicate, and two independent experiments were carried out.

2.2 Parasite invasion rate

Parasite invasion rate (pInvR) was defined as the number of tachyzoites invading the host cell at different time-points (hours) post-infection (hpi) and were determined following the methodology described in Dellarupe *et al*. (2014a) with minimal modifications. In order to obtain a confluent monolayer of F3 and BCEC-1, cells were seeded with 2×10^5 and 3×10^5 cells per well, respectively. Different densities of both cell types were used because F3 cells are bigger than BCEC-1 cells and formed a monolayer composed of polygonal cells while as BCEC-1 are smaller and they tended to form colonies and did not spread out the entire surface of the well. A total of 100 purified tachyzoites of each isolate were added to 24-well culture plates. Cultures were washed three times with phosphate buffered saline (PBS) at different time points (1, 2, 4, 6 and 8 hpi) for removing non-adhered/non-invading tachyzoites. Unwashed cultures were also included in the study. All plates were fixed at 48 hpi, and the pInvR was determined using single immunofluorescence staining as described below. To determine the pInvR, events (medium and large parasitophorous vacuoles) present in each well were counted using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 200×. The pInvR at 1, 2, 4, 6 and 8 hpi ($pInvR_{1h}$, $pInvR_{2h}$, $pInvR_{4h}$, $pInvR_{6h}$, $pInvR_{8h}$, respectively) was determined as the number of events per well in cell monolayers washed at different time points, and the total parasite invasion rate ($pInvR_T$) was determined as the number of events per well in unwashed cultures.

2.3 Cell infection rate

Multiplicity of infection (MOI) was defined as the ratio of the number of tachyzoites added to a known number of cells in a culture. Cell infection rate (cInfR) was defined as the percentage of cells infected using different MOIs (1, 2, 4, 6, 8 and 10). Cells were cultured in 24-well plates at concentration of $2 \times 10^5$and 3×10^5 cells per well for F3 and BCEC-1 cells, respectively. Infected cells were washed 3 times with PBS after 4 hpi to facilitate the synchronization of the cultures. Finally, cells were fixed at 48 hpi and stained using single immunofluorescence staining as described below.The overall number of cells, the number of infected cells and the number of cells containing more than one vacuole (multi-infected cells) were counted in 10 arbitrarily selected fields using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 200×. Counting of events was carried out on images taken with three different filters (white light for discrimination of cell limits, blue-DAPI for visualization of the nuclei and red-Alexa 594 for examination of the tachyzoites) using a Nikon DSL1 camera (Chiyoda, TYO, Japan) and overlaid using Photoshop® software (Adobe Systems Incorporated, Mountain View, CA, USA). A mean value of 50 cells was counted in each field.

2.4 Adhesion-invasion assay

An adhesion-invasion assay was performed in F3 and BCEC-1 cultures seeded at concentration of 2×10^5 and 3×10^5 cells per well, respectively. Cells were infected at a MOI of 2, and cultures were washed with PBS at 4 hpi to remove non-adherent extracellular tachyzoites. Cultures were immediately fixed, and double immunofluorescence staining was carried out following the protocol described below. Adhered extracellular tachyzoites (green- and red-stained) and intracellular tachyzoites (red-stained only) were counted using a fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 400×. A total of 1000 tachyzoites was counted in each coverslip. The percentage of intracellular tachyzoites (red-stained) respect to the total number of tachyzoites (intracellular and extracellular adhered tachyzoites) (green-stained) at 4 hpi was calculated.

2.5 Intracellular proliferation assays: Proliferation kinetics, doubling time and tachyzoite yield determinations

Proliferation kinetics of Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cells were determined by quantifying the number of tachyzoites at specific times (4, 10, 22, 34, 46, 58, 70 and 82 hpi) by real-time PCR (qPCR). Cells were cultured and infected as indicated above using a MOI of 2. Cultures were washed at 4 hpi and subsequently maintained at 37 °C in 5% CO_2. The samples were collected adding 200 µl of PBS, 180 µl of lysis buffer and 20 µl of proteinase K (Qiagen, Hilden, Germany) to each well at 4, 10, 22, 34, 46, 58, 70 and 82 hpi, transferred to a microcentrifuge tube and frozen at -80 °C prior to DNA extraction.

In parallel, replicates of cell cultures in coverslips were infected as described above and were labelled using double-immunostaining to study microscopically the proliferation kinetics of both isolates in F3 and BCEC-1 cells. Three coverslips were photographed for each condition using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan).

The doubling time (T_d) was defined as the period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding the lag phase (period without parasite multiplication) and the egress phase (Regidor-Cerrillo *et al*., 2011). The T_d was determined by applying non-linear regression analysis and an exponential growth equation using GraphPad (San Diego, CA, USA). We represented T_d for each isolate and each cell line as the average value obtained from all the determinations that revealed a linear regression, $R^2 \geq 0.95$.

The tachyzoite yield (TY_{58h}) was defined as the average value of the number of tachyzoites quantified by qPCR at 58 hpi for each isolate and cell line.

2.6 Immunofluorescence staining

Single immunofluorescence staining was carried out as specified previously (Dellarupe *et al*., 2014a) with minimal variations. Parasites in fixed cultures were stained using hyperimmune rabbit antiserum directed against *N. caninum* tachyzoites (1:1000) as a primary antibody and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 594 (red, Thermo Fisher Scientific, Waltham, MA, USA) as a secondary antibody. The nuclei were stained by washing the cells with a solution of 1:5000 DAPI in PBS.

Double immunofluorescence staining was carried out following the protocol described by Regidor-Cerrillo *et al*. (2011) with minimal modifications. Fixed plates were treated with 3% BSA in PBS for 30 min at 25 °C to block unspecific antibody binding and autofluorescence. Then, cultures were treated with a 1:1000 dilution of anti-tachyzoite hyperimmune rabbit antiserum and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 488 (green, Thermo Fisher Scientific, Waltham, MA, USA). After this step, only extracellular tachyzoites were labelled in green. After the first staining, cells were permeabilized using a solution of 0.25% Triton 100X in PBS 0.3% BSA (30 min, 37 °C). Later, cultures were treated again with a dilution of anti-tachyzoite hyperimmune rabbit antiserum as primary antibody (1:1000) and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 594 as secondary antibody (red, Thermo Fisher Scientific, Waltham, MA, USA). Therefore, intracellular tachyzoites were labelled only in red, while extracellular tachyzoytes resulted labelled in green and in red. The nuclei were stained by washing the cells with a solution of 1:5000 DAPI in PBS and the coverslips were embedded in Fluoroprep (BioMerieux, Marcy-l'Étoile, France).

2.7 DNA extraction and real-time PCR

Genomic DNA was extracted from cellular samples using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was eluted in a volume of 60 µl of molecular-grade water. Concentrations of DNA were determined for each sample using a nanophotometer (Nanophotomer®, Implen GmbH, Munich, Germany) and samples were diluted 1:4 in molecular-grade water. Quantification of *N. caninum* DNA was performed by real-time PCR using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The Nc-5 region was targeted as described elsewhere (Collantes-Fernández *et al*., 2002). Five µl of diluted DNA from each sample were used for the qPCR assays. The number of *N. caninum* tachyzoites was determined by interpolating the C_t values (cycle threshold value, which represents the fractional cycle number reflecting a positive PCR result) on a standard curve. The standard curve was designed for the quantification of 10^{-1}–10^4 tachyzoites according to Regidor-Cerrillo *et al*. (2011). To normalize the quantification of the parasites in each sample, a bovine β-actin standard curve was designed (from 64 ng of DNA per µl to 0.2 ng per µl). The results were expressed as the

relation between amounts of parasite DNA and cell DNA ($R^2 \geq 0.99$; slope values varied from -3.67 to -3.13).

2.8 Statistical analysis

The parametric one-way ANOVA test, followed by a Tukey's multiple comparisons test, was performed to investigate the influence of time on the pInvR and MOI in the cInfR, and the two-way ANOVA test, followed by a Tukey's multiple comparisons test, was performed to study the influence of the parasite isolate and the cell type on the pInvR and cInfR. A Chi-square test was carried out to investigate the differences in the percentages of intracellular tachyzoites at 4 hpi in both target cell types. Bonferroni correction was used to eliminate error associated with making multiple comparisons. Statistical significance was established as $P < 0.05$. Differences that showed P-values ≥ 0.05 and < 0.1 were considered to be trending towards statistical significance. GraphPad Prism 5 v.5.01 (San Diego, CA, USA) software was used to perform all statistical analyses and graphical illustrations.

3. Results

3.1 Parasite invasion rate (pInvR)

To investigate the impact of the placental cell type in parasite invasion, the pInvR was evaluated in trophoblasts and caruncular cells at different time points post-infection (1, 2, 4, 6 and 8 hpi). The pInvRs of the Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cells are shown in Fig. 1. The number of invaded tachyzoites for both isolates significantly increased until 4 hpi in both F3 (Nc-Spain7, ANOVA: $F_{(5,35)}=11.87$, $P<0.0001$; Nc-Spain1H, ANOVA: $F_{(5,35)}=7.211$, $P<0.0001$, followed by a Tukey's multiple comparisons test) and BCEC-1 cells (Nc-Spain7, ANOVA: $F_{(5,35)}=9.825$, $P<0.0001$; Nc-Spain1H, ANOVA: $F_{(5,35)}=9.156$, $P<0.0001$, followed by a Tukey's multiple comparisons test). From 4 hpi onwards, significant differences werenot observed.

Regarding the influence of the target cell type, a higher pInvR was observed in F3 cells compared to BCEC-1 cells from 6 hpi onwards for the Nc-Spain7 isolate (two-way ANOVA test: $F_{(3,168)}=27.25$, $P<0.0001$, followed by a Tukey's multiple comparisons test). No statistically significant differences were found when the pInvRs of Nc-Spain1H in both cell lines were investigated. The influence of the parasite isolate on the invasion of bovine trophoblasts and caruncular cells was also investigated by comparison of the pInvRs between the Nc-Spain1H and Nc-Spain7 isolates, assayed at different times of infection. Nc-Spain7 showed a pInvR significantly higher than Nc-Spain1H from 6 hpi onwards in F3 cells (two-way ANOVA test: $F_{(3,168)}=27.25$, $P<0.0001$ followed by a Tukey's multiple comparisons test) (Fig. 1a). However, no statistically significant differences in pInvR were found between isolates in BCEC-1 cells (Fig. 1b).

3.2 Cell infection rate (cInfR)

The percentage of infected cells (cInfR) and the percentage of multi-infected cells were evaluated at different MOIs. The number of infected cells significantly increased with increasing MOIs in both cell lines, F3 (Nc-Spain7, ANOVA: $F_{(5,42)}=228.5$, $P<0.0001$; Nc-Spain1H, ANOVA: $F_{(5,42)}=273.4$, $P<0.0001$, followed by a Tukey's multiple comparisons test) and BCEC-1 (Nc-Spain7, ANOVA: $F_{(5,42)}=30.04$, $P<0.0001$; Nc-Spain1H, ANOVA: $F_{(5,42)}=42.60$, $P<0.0001$, followed by a Tukey's multiple comparisons test). The cInfRs were higher in infected F3 than in BCEC-1 cells for both Nc-Spain7 and Nc-Spain1H isolates at the same MOI (Fig. 2a,b) (two-way ANOVA test: $F_{(3,168)}=222.4$, $P<0.0001$, followed by a Tukey's multiple comparisons test). The percentage of cells containing more than a single vacuole (Fig. 2c, d) was also higher in F3 than in BCEC-1 infected by both isolates (two-way ANOVA test: $F_{(3,168)}=93.64$, $P<0.0001$, followed by a Tukey's multiple comparisons test).

In addition, Nc-Spain7 showed a higher cInfR than Nc-Spain1H in both cell lines at different MOIs (two-way ANOVA test: $F_{(3,168)}=222.4$, $P<0.0001$, followed by a Tukey's multiple comparisons test) (Fig. 2a, b). We also observed that Nc-Spain7 showed a higher percentage of multi-infected cells than Nc-Spain1H in F3 cells at 6, 8 and 10 MOIs (two-way ANOVA test: $F_{(3,168)}=93.64$, $P<0.0001$ followed by a Tukey's multiple comparisons test) (Fig. 2c). However, no significant differences in the number of multi-infected cells were found between isolates in BCEC-1 cells (Fig. 2d).

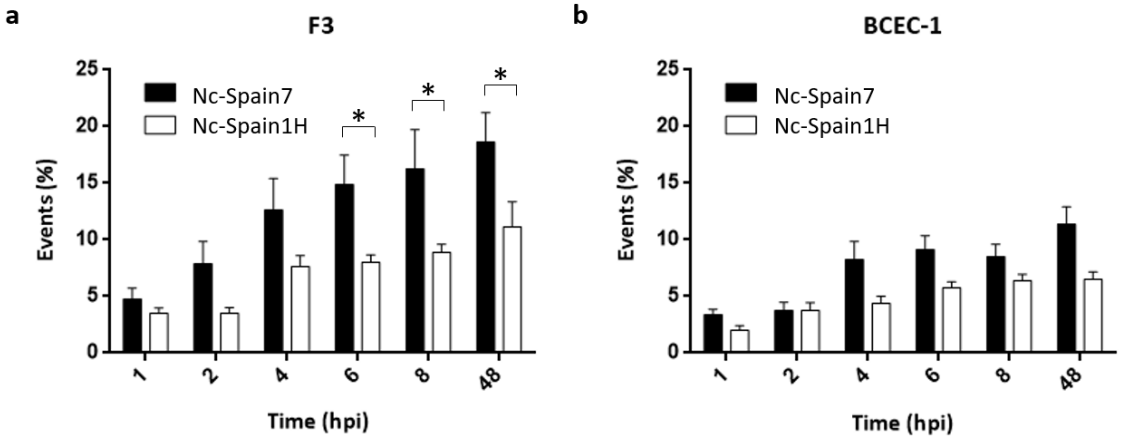


Figure 1. Parasite invasion rates in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H isolates. Graphs represent parasite infection rates in F3 (**a**) and BCEC-1 cells (**b**) defined as the percentage of invaded tachyzoites (number of events per well) studied at different time points for Nc-Spain7 and Nc-Spain1H. Each column and error bar represent the mean and the SD of 4 replicates from 2 independent assays at the indicated sampling times. The total number of invaded tachyzoites was determined by single immunofluorescence staining of events (parasitophorous vacuoles and lysis plaques) followed by counting using an inverted fluorescence microscope. Significantly higher pInvRs were found in F3 cells compared to BCEC-1 cells infected with Nc-Spain7 ($P < 0.01$), whereas no differences were found in the pInvRs of F3 and BCEC-1 cells infected by Nc-Spain1H ($P > 0.05$). * represents significant differences between isolates

3.3 Adhesion-invasion assay

In the light of the differences in pInvR and cInfR between both cells lines, an adhesion-invasion assay was performed to investigate whether these differences could be attributed to a different adhesion ability of the tachyzoites in these two cell lines, a different ability to penetrate in the cells or both (Fig. 3a). In this assay, non-adhered tachyzoites were eliminated in the washing step at 4 hpi before the fixation and extra- and intracellular adhered tachyzoites were counted. The percentage of intracellular tachyzoites respect to the total adhered intra- and extracellular tachyzoites was calculated. Surprisingly, both isolates showed that almost 100% of adhered tachyzoites were intracellular at 4 hpi in BCEC-1 cells, whereas a minor percentage of intracellular tachyzoites was observed in F3 cells, 88 and 69% for Nc-Spain7 and Nc-Spain1H, respectively. A significantly higher number of adhered tachyzoites from both isolates were internalized in BCEC-1 cells than in F3 cells at 4 hpi (Chi-square test: $\chi^2 = 287.6$, $df = 3$, $P < 0.0001$) (Fig. 3b).

Differences between isolates were not observed in BCEC-1, although the high-virulence isolate Nc-Spain7 showed a better ability to penetrate than the low-virulence isolate Nc-Spain1H in F3 cells (Fisher's exact test: $P < 0.0001$) (Fig. 3b).

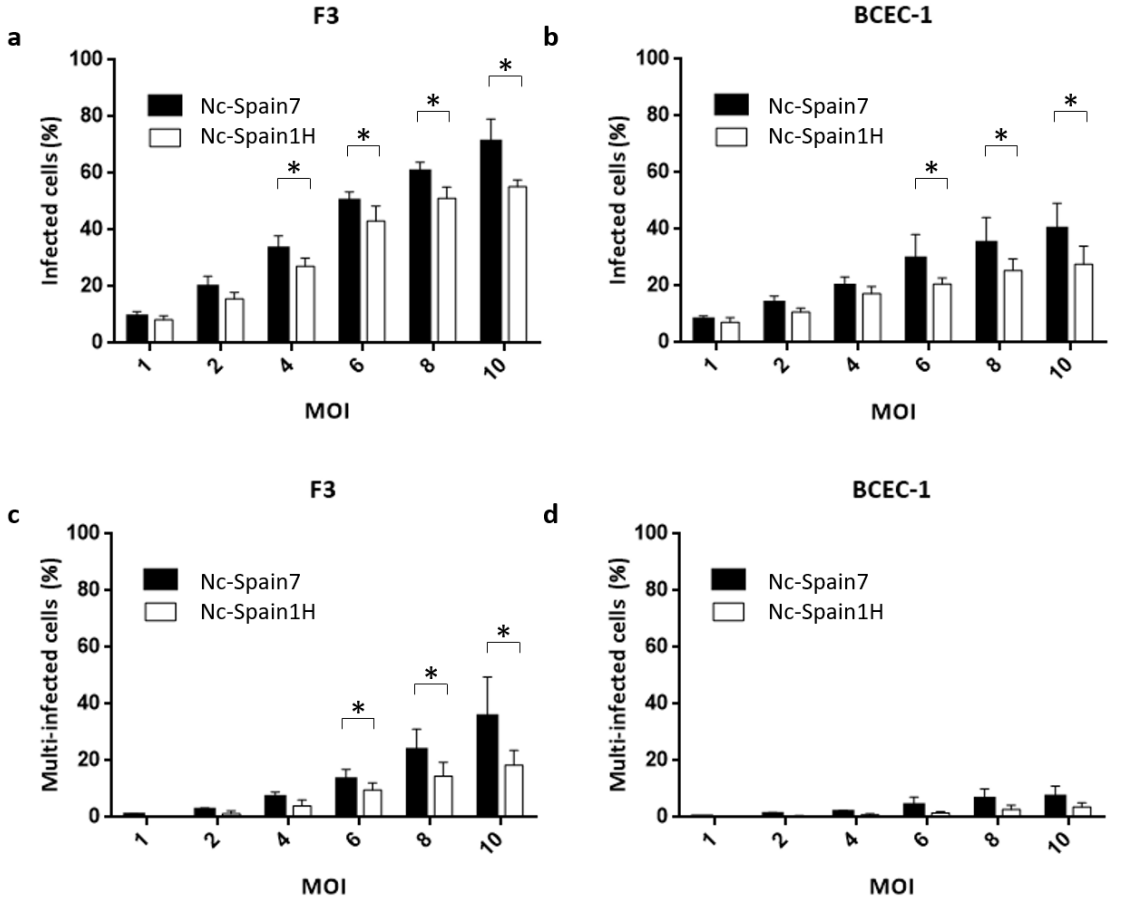


Figure 2. Infection and multi-infection rates in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H isolates. Graphs represent the cell infection rates as the percentage of infected cells in F3 (**a**) and BCEC-1 cells (**b**) for both isolates and the percentage of cells with multi-infection (more than one parasitophorous vacuole) in F3 (**c**) and BCEC-1 cells (**d**). Each column and error bar represents the mean and the SD of 4 replicates from 2 independent assays using different MOIs. The total number of cells, the number of infected cells and the number of cells with multi-infection were determined by double immunofluorescence staining followed by counting using an inverted fluorescence microscope. The cInfRs were higher in F3 than in BCEC-1 cells infected by both isolates ($P < 0.0001$). The percentage of cells containing more than a single vacuole was also higher in F3 than in BCEC-1 cells infected by both isolates ($P < 0.05$). * represents significant differences between isolates

3.4 Proliferation kinetics, doubling time comparisons and tachyzoite yield determination

An *in vitro* intracellular proliferation assay was carried out to study proliferation and egress events of the lytic cycle of *N. caninum* in trophoblast and caruncular cell cultures. Proliferation kinetics over time assessed by qPCR are represented in Fig. 4a, b. The growth curves of both isolates in F3 and the growth curve of Nc-Spain7 in BCEC-1 adjusted to exponential growth from 10 hpi to 70 hpi, whereas the growth curve of Nc-Spain1H in BCEC-1 did not adjust either to the exponential or linear growth mathematical model. Analysing the T_d, we observed a delay in the multiplication of *N. caninum* in BCEC-1 cells, with the average T_d value of Nc-Spain7 1.5-times higher in BCEC-1 cells (14.603±1.428) than in F3 cells (9.425±0.239) (one-way ANOVA: $F_{(2,21)}=6.966$, $P=0.0048$ followed by a Tukey's multiple comparisons test). The Nc-Spain1H isolate showed an average T_d value of 12.246±0.893 in F3 cells. The T_d value for Nc-Spain1H in BCEC-1 could not be calculated due to the lack of exponential growth of Nc-Spain1H in BCEC-1. Nevertheless, no significant differences were found in the average T_d values for Nc-Spain7 and Nc-Spain1H isolates in F3 cells.

A microscopic examination of cultures fixed at different time points showed that the multiplication of Nc-Spain7 and Nc-Spain1H isolates began between 10 and 22 hpi. Differences in the parasitophorous vacuole size between both isolates were observed in F3 cells from 34 hpi onwards, with

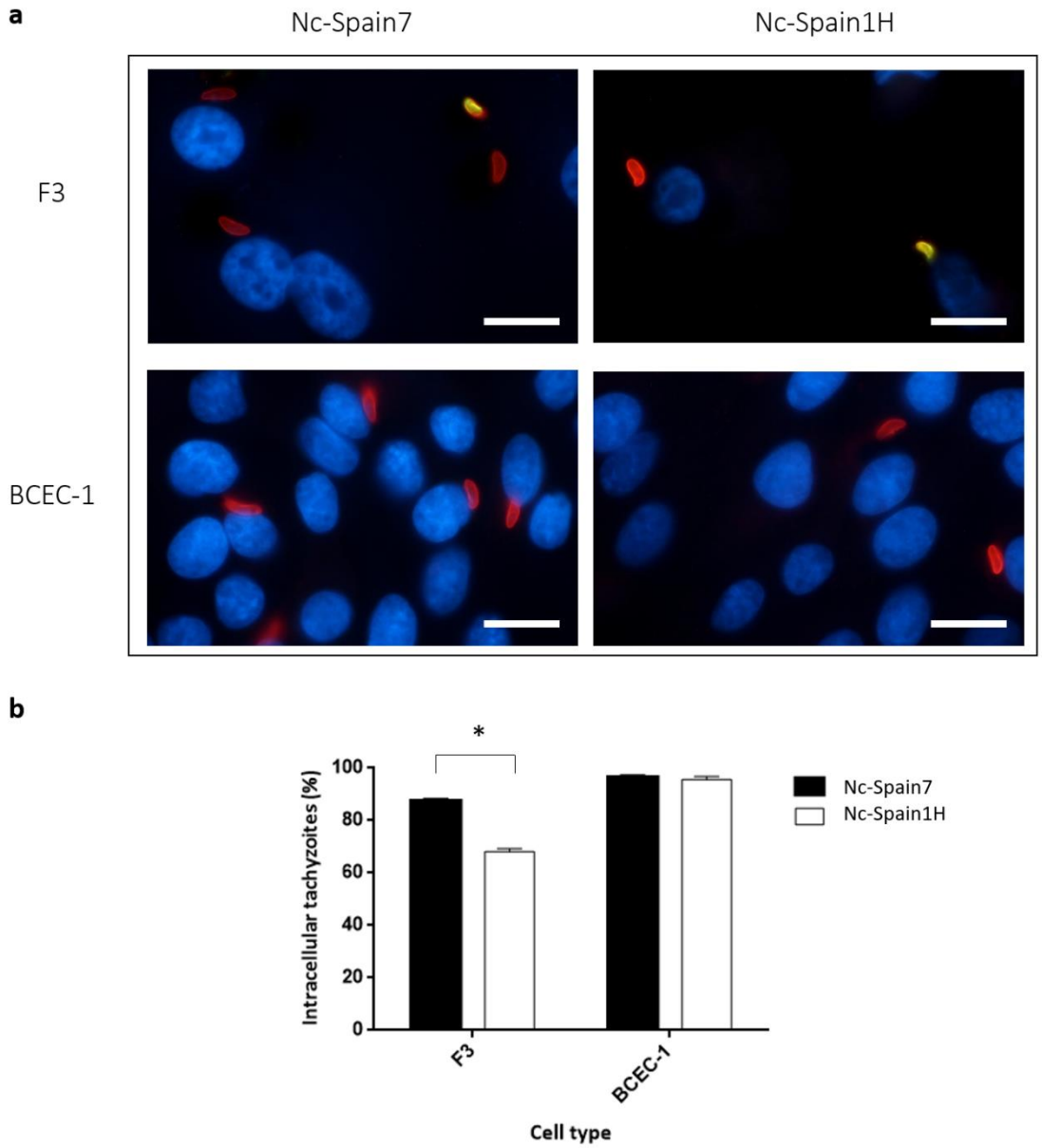


Figure 3. Adhesion assay in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H at 4 hpi. Double immunofluorescence staining was performed and adhered extracellular tachyzoites were stained with Alexa Fluor® 488 (green) and Alexa Fluor® 594 (red), whereas intracellular tachyzoites were stained with Alexa Fluor® 594 (red). Nuclei were stained with DAPI (blue). Tachyzoites were counted in 10 arbitrarily selected fields, and the percentage of intracellular tachyzoites relative to the number of total adhered tachyzoites at 4 hpi was calculated. Representative images at a magnification of 1000× (**a**) show the adhesion assay performed in F3 and BCEC-1 cells infected with both isolates. The graph (**b**) represents the percentage of intracellular tachyzoites of Nc-Spain7 and Nc-Spain1H relative to the total number of intra- and extracellular tachyzoites adhered to F3 and BCEC-1 cells. Each column and error bar represent the mean and the SD of 4 replicates from 2 independent assays. BCEC-1 cells showed a significantly higher percentage of intracellular tachyzoites than F3 cells ($P < 0.0001$, Chi-square test). The percentage of intracellular tachyzoites for Nc-Spain7 (88%) was significantly higher than for Nc-Spain1H (69%) in F3 ($P < 0.0001$), whereas the percentage of intracellular tachyzoites of both isolates in BCEC-1 was the same (96%). * represents significant differences between isolates. Scale-bars: **a**, 40 µm

bigger vacuoles in Nc-Spain7 infected cells. However, no differences between the isolates were demonstrated by immunofluorescence in BCEC-1 cells. Between 58 and 82 hpi, asynchronous rupture of host cells and egress of the tachyzoites were observed in F3 cells. However, interestingly, an early egression of tachyzoites from 22 hpi onwards was observed in BCEC-1 (Fig. 4c).

The TY_{58h} was assessed to determine the number of tachyzoites produced during the same intracellular period after invasion, prior to complete tachyzoite egress from cell cultures (Fig. 4d). The TY_{58h} was 15-times higher in F3 cells than in BCEC-1 cells infected with Nc-Spain7, and 10-times higher in F3 cells infected with Nc-Spain1H. Comparing the isolates, Nc-Spain7 showed a higher TY_{58h} than Nc-Spain1H in F3 (one-way ANOVA: $F_{(3,28)} = 37.35$, $P < 0.0001$ followed by a Tukey's multiple comparisons test), whereas no differences in the TY_{58h} were found in BCEC-1.

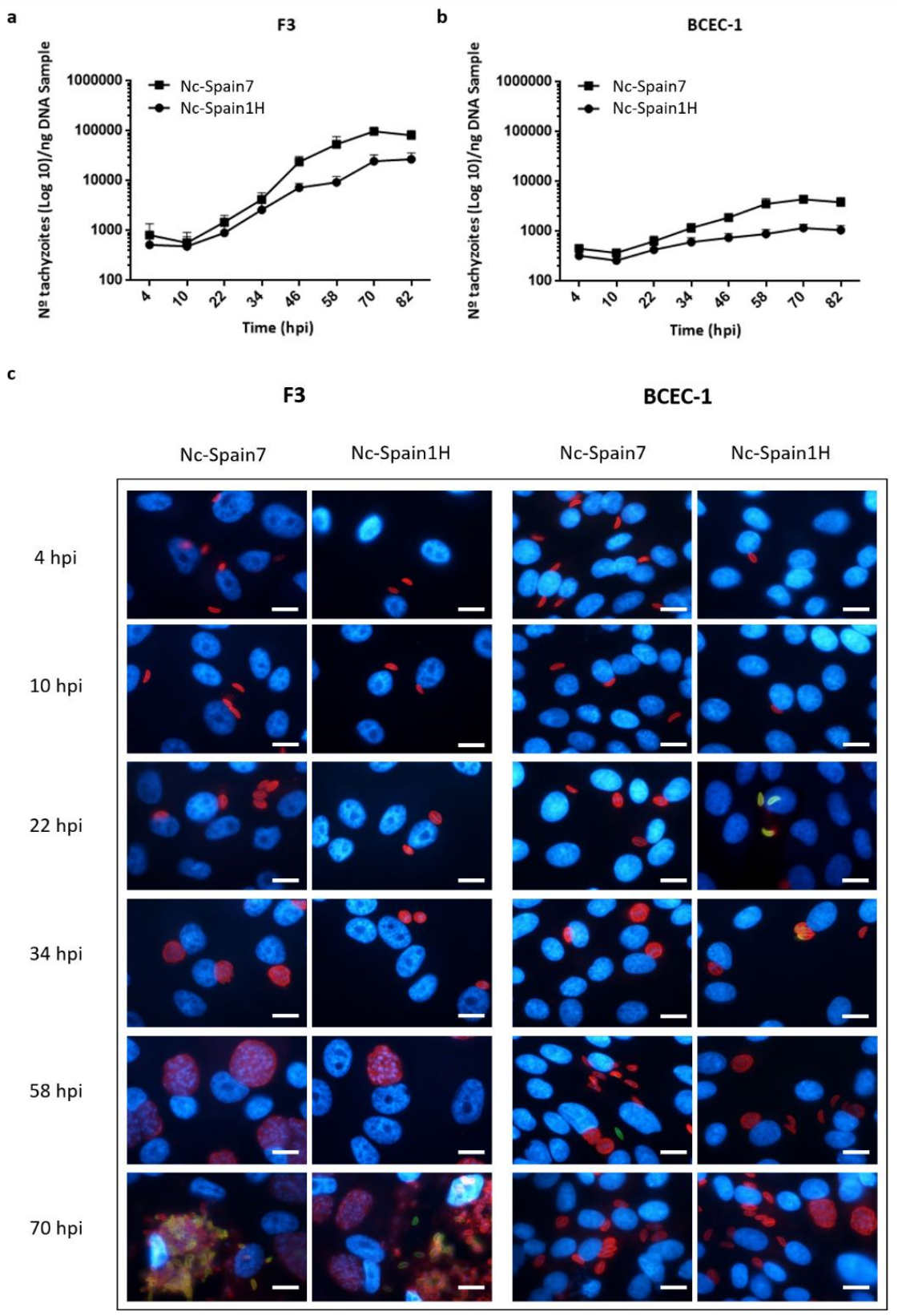
The results obtained in the present work are summarized in Table 1.

4. Discussion

In the present study, we established for the first time a species- and organ-specific *in vitro* model for each of the host cell layers in the maternal-foetal interface of the bovine placenta to study *N. caninum* infection. To date, several *in vitro* studies have been carried out using established cell lines, such as Marc-145, HeLa, BeWo or ovine trophoblast cells, to investigate the invasion and proliferation of different *N. caninum* isolates (Haldorson *et al.*, 2005; Carvalho *et al.*, 2010; Regidor-Cerrillo *et al.*, 2011). There is only one (limited) descriptive study concerning the interaction of the parasite with bovine trophoblast cells (Machado *et al.*, 2007), and no data are available about the parasite's interaction with bovine caruncular cells. The interaction between *N. caninum* and these target cells has been studied in the present work, using two isolates of different virulence and the two cell lines that represent the maternal-foetal interface. The cell lines used in this work (bovine trophoblast cells, F3, and bovine caruncular epithelial cells, BCEC-1) were isolated from fifth- and fourth-month pregnant heifers, respectively, and they have maintained at least part of their morphological and functional characteristics (Bridger *et al.*, 2007b; Hambruch *et al.*, 2010; Waterkotte *et al.*, 2011). Thus, they may be a useful tool to investigate the pathways of *N. caninum* infection during transplacental transmission during the second trimester of pregnancy when the majority of abortions caused by *N. caninum* occur (Dubey *et al.*, 2007). Investigations were focused on the lytic cycle of the tachyzoites (host-cell invasion, proliferation and egress). The processes implicated in the lytic cycle of the parasite are essential for the invasion of host tissues, the distribution of the parasite through the organism and its distribution to the placenta. As a consequence, abortion or transplacental transmission may occur (Hemphill *et al.*, 2006; Dubey *et al.*, 2007). Here, two *N. caninum* isolates with marked differences in virulence were able to establish themselves and multiply both in the maternal epithelium of the caruncle and in the foetal trophoblast, although differences in the infection of both cell types were found.

Our results showed a lower infection rate, as well as a lower percentage of cells with multi-infection, for both parasite isolates in BCEC-1 cells relative to F3 cells. Therefore, *N. caninum* tachyzoites more efficiently infect trophoblast cells compared to bovine caruncular epithelial cells, meaning bovine trophoblast cells are more susceptible to *N. caninum* infection. In experimental infections, higher parasite burdens and more severe lesions have been found in the foetal part of the placenta (Caspe *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014). As previously demonstrated, BCEC-1 is an established cell line showing *in vitro* characteristics of a morphologically and functionally intact epithelial barrier with apical microvilli and junctional complexes (zonula occludens, zonula adherens and desmosomes) (Bridger *et al.*, 2007a; Bridger *et al.*, 2007b) as described *in vivo* (Björkman, 1973; Leiser, 1975). This polarized barrier, with apical junctional complexes obliterating the paracellular space, establishes an effective paracellular barrier to diffusion of fluid and solutes, limiting the passage of foetal and maternal metabolites (Bridger, 2008).

These characteristics may be hindering the paracellular passage of *N. caninum* across the epithelium. Foetal cells (F3) share many properties with maternal cells (BCEC-1), including apical microvilli and expression of the tight junctional zonula occludens protein both *in vivo* (Björkman, 1973; Leiser, 1975) and *in vitro* (Hambruch *et al.*, 2010); however, in contrast to the maternal BCEC-1 cells, mononuclear trophoblast cells have phagocytic phenotypes *in vivo* (Schlafer *et al.*, 2000). Also bovine F3 cells may form binucleated cells (Hambruch *et al.*, 2010), which have


a
F3
Nº tachyzoites (Log 10)/ng DNA Sample
1000000
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100
Nc-Spain7
Nc-Spain1H
4
10
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34
46
58
70
82
Time (hpi)
b
BCEC-1
Nº tachyzoites (Log 10)/ng DNA Sample
1000000
100000
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1000
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Nc-Spain7
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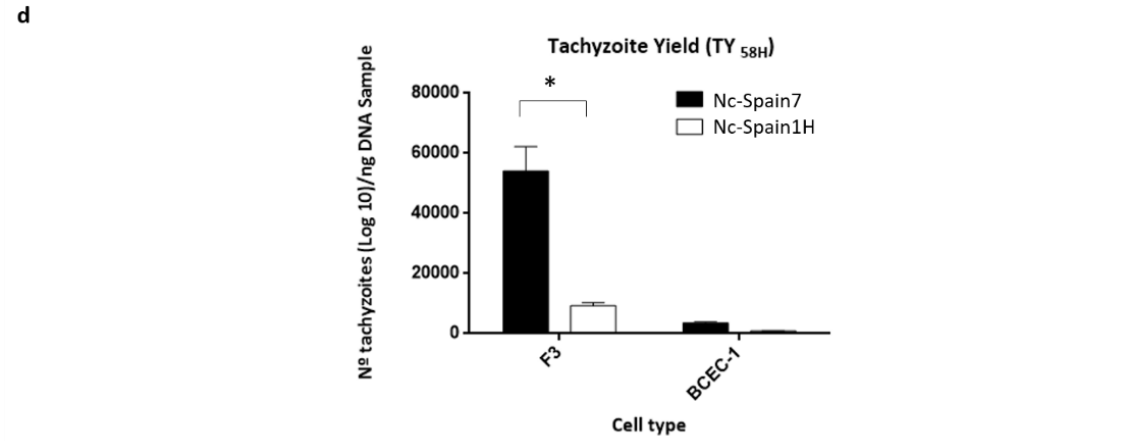

Figure 4. Proliferation kinetics over time and tachyzoite yield at 58 hpi. Graphs (a and b) represent the average number of tachyzoites for each time-point for all individual experiments with an $R^2 > 0.95$, except for BCEC-1 cells infected by Nc-Spain1H, which showed a nonexponential growth pattern. Error bars indicate the SD. Representative images (c) show the proliferation kinetics over time of Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cultures. The bar graph (d) represents the tachyzoite yield at 58 hpi for Nc-Spain7 and NcSpain1H in F3 and BCEC-1 cells. Each column and error bar represent the mean and the SD of 4 replicates from 2 independent assays. The TY58h was fifteen times higher in F3 cells than in BCEC-1 cells infected with Nc-Spain7, and ten times higher in F3 cells infected with Nc-Spain1H. Statistical differences were found in the TY58h between isolates in F3 cells, with the TY_{58h} of Nc-Spain7 significantly higher than for Nc-Spain1H ($P < 0.0001$). * represents significant differences between isolates. Scale-bars: c, 10 µm

phagocytic activity as has been previously described for trophoblast giant cells of various species (Schlafer *et al*., 2000; Amarante-Paffaro *et al*., 2004; Bevilacqua *et al*., 2010). The phagocytic activity of mono- and binucleate trophoblast cells may be mediating parasite passage to the foetus (Machado *et al*., 2007). In fact, in a BALB/c mouse model infected with *T. gondii*, a higher frequency of infected placentas was observed at later stages of pregnancy, which has been correlated with a higher phagocytic efficiency of the placental tissues in this period (Wujcicka *et al*., 2014). Therefore, although differences in junctional complexes between both cell types should be investigated to evaluate their influence in parasite invasion, it seems probable that the phagocytic ability of trophoblast cells may partially explain the higher susceptibility to parasite invasion observed in these cells.

Noting the differences in the invasion and infection rates between both cell lines, an adhesion-invasion assay was performed in order to elucidate whether these differences could be associated with lower adhesion, penetration or both. Our results revealed that, contrary to expectations, both isolates, which had presented lower cell infection rate in BCEC-1 cells compared to F3 cells, showed a highly efficient invasion with almost 100% penetration of the adhered tachyzoites in BCEC-1 cells at 4 hpi. Thus, the lower invasion of *N. caninum* observed in BCEC-1 cells may be due to a lower ability to adhere or to a fragile adhesion to host-cell receptors. Tachyzoite adhesion occurs in two phases, as previously described (Hemphill *et al*., 2006). The first step is the establishment of low-affinity contact between tachyzoites and the host-cell surface membrane, where surface antigens of *N. caninum* tachyzoites such as NcSAG1 and NcSRS2 are involved. Later, the actual adhesion process occurs via to microneme proteins (especially NcMIC3), which bind to host-cell surface chondroitin sulfates. Studying the differences in the type and abundance of superficial receptors responsible for the high-affinity interaction with tachyzoites between both cell lines could aid the understanding of the diminished adhesion of *N. caninum* in bovine caruncular cells.

Concerning the growth kinetics of *N. caninum* in foetal and maternal cells, our results showed a dramatically lower proliferation of both isolates in caruncular cells. Moreover, Nc-Spain1H did not adjust to an exponential growth in maternal cells. Differences observed between both cell types may be partially attributed to a different degree of maturation. While the foetus is not completely immunocompetent in the second trimester of gestation, maternal cells have immunocompetent abilities that may restrict the infection and proliferation of the parasite. *In vivo* studies have demonstrated the influence of the gestational stage

Table 1. Summary of virulence traits as a function of cell type and *N. caninum* isolate

		pInvR	cInfR	% Multi-infection	Invasion efficiency	TY_{58H}	Td
Cell type comparisons *(F3 vs BCEC-1)*	Nc-Spain7	+	++++	+	----	+	--
	Nc-Spain1H	NS	++++	+	----	+	NC[g]
Isolate comparisons *(Nc-Spain7 vs Nc-Spain1H)*	F3	++	+	++++	++++	++	NS
	BCEC-1	NS	+	NS	NS	NS	NC[g]

[a]pInvR (parasite invasion rate): number of tachyzoites invading the host cell at different time points post-infection
[b]cInfR (cell infection rate): percentage of cells infected using different parasite doses
[c]% Multi-infection: percentage of cells containing more than one vacuole
[d]Invasion efficiency: results from adhesion-invasion assay, percentage of intracellular tachyzoites relative to the total number of tachyzoites at 4 hpi
[e]TY_{58H} (tachyzoite yield at 58 hpi): average number of tachyzoites quantified by qPCR at 58 hpi
[f]T_d (Doubling time): period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding lag and egress phases
[g] *NC* data not comparable. The T_d value for Nc-Spain1H isolate in BCEC-1 cells could not be calculated due to the lack of exponential growth of Nc-Spain1H in BCEC-1
[h] *NS* no significant differences
+/++/++++ indicate higher rates of each parameter assayed with a significance of $P < 0.05$, $P < 0.01$, $P < 0.0001$, respectively
-/--/---- indicate lower rates of each parameter assayed with a significance of $P < 0.05$, $P < 0.01$, $P < 0.0001$, respectively

on the outcome of *N. caninum* infection (Williams *et al*., 2000; Malet *et al*., 2003; Macaldowie *et al*., 2004; Collantes-Fernández *et al*., 2006). It is known that the survival of the foetus depends on the state of development of its immune system, as higher abortion rates, higher parasite burdens and more severe lesions were observed in foetal tissues when infection occurred in the first and second trimester of gestation (Williams *et al*., 2000; Macaldowie *et al*., 2004; Collantes-Fernández *et al*., 2006c). On the other hand, the lower multiplication of *N. caninum* in the maternal side of the placenta supports the hypothesis that caruncles act as a barrier, limiting not only parasite infection via reduced adhesion but also its multiplication. In experimental infections, comparisons between cytokine mRNA levels in separated maternal and foetal placental tissues showed that maternal tissue was the major source of most cytokines (Rosbottom *et al*., 2008) and had a major lymphocyte cell infiltration, particularly in the maternal caruncle (Gibney *et al*., 2008; Rosbottom *et al*., 2011), which may indicate that the maternal immune system was actively responding to the parasite. Moreover, early egress was observed in caruncular cells, which could be employed by the parasite as an escape mechanism to facilitate the dissemination of the parasite to the foetal part of the placenta, which has been demonstrated in this work to be the parasite's preferential target cell, and, thus, allow vertical transmission of the parasite.

As mentioned above, the role of the parasite in the outcome of infection is also a determining factor. Processes involved in the lytic cycle, including parasite invasion and intracellular proliferation, are essential for the maintenance and multiplication of the parasite *in vitro* and for parasite survival and propagation in host tissues during the course of animal infection (Hemphill *et al*., 2006; Dubey *et al*., 2007). In previous studies, several *N. caninum* isolates showed differences during the *in vitro* lytic cycle and more virulence than others in animal models, associated with higher abortion and transmission rates (Rojo-Montejo *et al*., 2009a;Rojo-Montejo *et al*., 2009b; Pereira García-Melo *et al*., 2010; Regidor-Cerrillo *et al*., 2010; Regidor-Cerrillo *et al*., 2011; Regidor-Cerrillo *et al*., 2014). In trophoblast cells, both isolates, described as "highly prolific" (Nc-Spain7) and "less prolific" (Nc-Spain1H) in previous studies (Regidor-Cerrillo *et al*., 2011), showed the same *in vitro* characteristics. In particular, the virulent isolate Nc-Spain7 demonstrated greater invasion, infection and proliferation rates than Nc-Spain1H in trophoblast cells. These differences may be explained by their biological diversity, as has been demonstrated in previous *in vivo* studies (Rojo-Montejo *et al*., 2009a; Rojo-Montejo *et al*., 2009b;

Regidor-Cerrillo *et al*., 2011; Regidor-Cerrillo *et al*., 2012; Regidor-Cerrillo *et al*., 2013; Regidor-Cerrillo *et al*., 2014; Regidor-Cerrillo *et al*., 2015). Nc-Spain7 showed a high neonatal mortality (95%) and vertical transmission rate (nearly 80%) in a pregnant BALB/c mouse model (Regidor-Cerrillo *et al*., 2010), as well as a percentage of abortion and vertical transmission as high as 100% in a bovine model (Regidor-Cerrillo *et al*., 2014; Almería *et al*., 2016a). However, Nc-Spain1H showed a 100% offspring survival rate and a low vertical transmission rate (5%) in a pregnant mouse model (Rojo-Montejo *et al*., 2009a), and no foetal death was observed in experimentally infected cattle (Rojo-Montejo *et al*., 2009b). The higher proliferation ability of Nc-Spain7 in trophoblast cells found in the present study may be responsible for the increase in the quantity of parasites reaching the foetal tissues and, consequently, for the enhancement of parasite burdens and pathology, ultimately resulting in foetal death and abortion. These results agree with those obtained in previous studies, where higher parasite burdens in the brain and placental tissues, a wider spread and greater severity of histopathological lesions and clinical signs were observed in animals experimentally infected with Nc-Spain7 (Rojo-Montejo *et al*., 2009b; Pereira García-Melo *et al*., 2010; Regidor-Cerrillo *et al*., 2014). However, in Nc-Spain1H-infected animals, less severe lesions were observed in placentas and maternal and foetal tissues (Rojo-Montejo *et al*., 2009b), which may explain the absence of abortion. In terms of dissemination *in vivo*, isolates with low virulence could have a lower efficiency at crossing biological barriers.

More interestingly, contrary to our observation in trophoblast cells, the behaviour of both isolates was very similar in bovine caruncular cells. Differences between isolates were limited to a slightly higher infection rate by the virulent isolate Nc-Spain7, whereas adhesion, invasion and proliferation mechanisms were very similar for both isolates. This fact has also been observed in the phylogenetically-related protozoan *T. gondii*, where comparisons between three strains showed no significant differences in their capacity to infect human placental explants (Robbins *et al*., 2012). The comparable behaviour showed by different virulence isolates, together with the lower invasion, infection and proliferation rates found in caruncular cells, leads us to hypothesize that isolates may have been selected because of a low virulence in the maternal part of the placenta despite their differences in virulence traits in other host cells, including other placental cells such as trophoblasts. This reduced virulence in the caruncle may facilitate, on the one hand, evasion from maternal immunity and the placental damage caused by parasite multiplication, leading to the abortion. On the other hand, this behaviour may facilitate vertical transmission to the progeny, which is the main route of transmission for *N. caninum.* In fact, Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy but congenitally infected calves, as described above.

5. Conclusions

This is the first study where an *in vitro* model of *N. caninum* infection has been implemented in bovine placental cells. Our findings confirm a differential competency of two isolates of *N. caninum* with different virulence to proliferate in bovine trophoblast cells. However, bovine caruncular cells were the first cell line assayed where different virulence isolates showed similar invasion, adhesion and proliferation kinetics. The low replication of both isolates in the maternal side of the placenta may facilitate the evasion of the immune response by the parasite, allowing their transplacental transmission. This fact may have constituted an evolutionary advantage for these isolates. Remarkably, limited parasite invasion and growth in caruncular cells suggest a putative barrier function for this cell type in the placenta, although early parasite egress may facilitate transmission to offspring.

Furthermore, our results confirm the role of foetal trophoblasts as target cells for *N. caninum*. Future research to determine the differences in surface receptors and cell junctions between both placental cell types are needed. In addition, studies focused on co-cultures of maternal and foetal cells may be helpful as a model to study parasite transport across the maternal epithelium as part of the bovine placental barrier. Finally, the existence of differences in local immunomodulation and cellular mechanisms that take place in the placenta infected by high- and low-virulence isolates of *N. caninum* should be investigated.

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Authors' contributions
JRC, PH, ECF, MGB and LMO conceived the study and participated in its design. LJP and MGS wrote the manuscript, with interpretation of results and discussion inputs from JRC, ECF, MGB, LMO, NH and CP. LJP and MGS performed *in vitro* plaque and immunofluorescence assays. JRC, PH, LJP and MGS designed and performed RT-qPCR analyses. NH and CP isolated bovine trophoblast and caruncular cell lines used in the assays. LJP and MGS carried out statistical analyses and interpreted the results. All authors read and approved the final manuscript.

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Not applicable.

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The authors declare that they have no competing interests.

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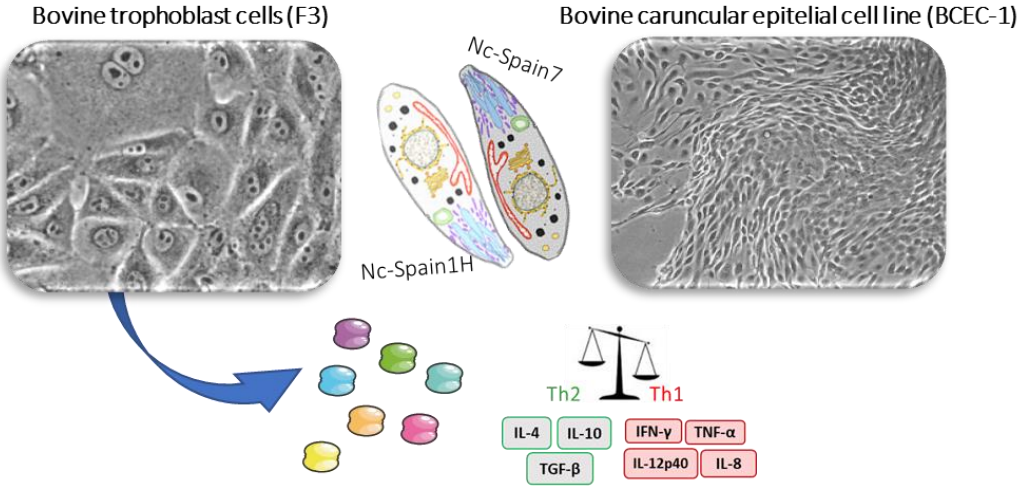
Immune response profile of caruncular and trophoblast cell lines infected by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *Neospora caninum*

Laura Jiménez-Pelayo[1†], Marta García-Sánchez[1†], Javier Regidor-Cerrillo[1], Pilar Horcajo[1], Esther Collantes-Fernández[1], Mercedes Gómez-Bautista[1], Nina Hambruch[2], Christiane Pfarrer[2] and Luis Miguel Ortega-Mora[1*]

[1]SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[2]Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

[†]Authors contributed equally



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Abstract

Background: Bovine neosporosis, one of the main causes of reproductive failure in cattle worldwide, poses a challenge for the immune system of pregnant cows. Changes in the Th-1/Th-2 balance in the placenta during gestation have been associated with abortion. Cotyledon and caruncle cell layers form the maternal-foetal interface in the placenta and are able to recognize and induce immune responses against *Neospora caninum* among other pathogens. The objective of the present work was to elucidate the immunomodulation produced by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *N. caninum* in bovine trophoblast (F3) and caruncular cells (BCEC-1) at early and late points after infection. Variations in the mRNA expression levels of toll-like receptor-2 (TLR-2), Th1 and Th2 cytokines (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17, IFN-γ, TGF-β1, TNF-α), and endothelial adhesion molecules (ICAM-1 and VCAM-1) were investigated by RT-qPCR, and protein variations in culture supernatants were investigated by ELISA.

Results: A similar pattern of modulation was found in both cell lines. The most upregulated cytokines in infected cells were pro-inflammatory TNF-α (P<0.05–0.0001) and IL-8 (P<0.05–0.001) whereas regulatory IL-6 (P<0.05–0.001) and TGF-β1 (P<0.05–0.001) were downregulated in both cell lines. The measurement of secreted IL-6, IL-8 and TNF-α confirmed the mRNA expression level results. Differences between isolates were found in the mRNA expression levels of TLR-2 (P<0.05) in both cell lines and in the mRNA expression levels (P<0.05) and protein secretion of TNF-α (P<0.05), which were higher in the trophoblast cell line (F3) infected with the low-virulence isolate Nc-Spain1H.

Conclusions: *Neospora caninum* infection is shown to favour a pro-inflammatory response in placental target cells *in vitro*. In addition, significant immunomodulation differences were observed between high- and low-virulence isolates, which would partially explain the differences in virulence.

Keywords: *Neospora caninum*, Cattle, Immune response, Placenta, Trophoblast, Caruncle, Isolates, Virulence, Cytokines

1. Background

Bovine neosporosis is one of the main transmissible causes of abortion in cattle worldwide (Innes *et al*., 2005; Dubey *et al*., 2006; Dubey *et al*., 2007). The etiological agent of bovine neosporosis is *Neospora caninum*, an obligate intracellular parasite closely related to the zoonotic agent *Toxoplasma gondii*. Transplacental transmission is the main route of transmission in cattle (Williams *et al*., 2009) and the placenta can play a key role in the pathogenesis of bovine neosporosis (Entrican, 2002; Innes, 2007). The direct damage produced by the multiplication of the parasite in placental and foetal tissues has been proposed as one of the possible causes of abortion observed during *N. caninum* infections. Importantly, the placenta is considered to be an immune regulatory organ since it acts as a modulator of foetal and maternal immune responses. In fact, an immune-mediated pathogenesis has also been suggested as a possible cause of abortion (Quinn *et al*., 2002b). It has been shown that the multiplication of the parasite in the placenta alters the immunological balance at the maternal-foetal interface with an increase of local pro-inflammatory IFN-γ, IL-12p40 and TNF-α cytokines which could compromise the gestation, together with an increase in IL-4 and IL-10 levels (Rosbottom *et al*., 2008; Regidor-Cerrillo *et al*., 2014), which avoids the immunological rejection of the foetus but favours the multiplication and vertical transmission of the parasite (Entrican, 2002; Innes *et al*., 2002). Trophoblast and caruncular cells are able to recognize pathogens and secrete cytokines and chemokines that recruit immune cells in the damaged area (Montes *et al*., 1995; Steinborn *et al*., 1998a; Steinborn *et al*., 1998b). Thus, both cell types play a fundamental role in the initiation of innate immune responses at the placental level as well as in the development of an adaptative immune response for the pregnant dam and foetus.

Previous *in vivo* studies have shown the influence of the isolate on the dynamics and outcome of the infection in pregnant bovine models and in the cytokine profiles induced during the infection (Rojo-Montejo *et al*., 2009b; Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014; Dellarupe *et al*., 2014b, Jiménez-Pelayo *et al*. unpublished data). To date, only one recent study has utilized an *in vitro* model consisting of immortalized bovine trophoblasts (F3) from the foetus and caruncular cells (BCEC-1) from the dam. The aim of the study was to elucidate the interactions between tachyzoites and the host cells that resemble the maternal-foetal interface of the bovine placentome while also taking into account the influence of the isolate. Maternal cells, where both isolates showed similar phenotypic traits, presented higher resistance to the infection than trophoblast cells, where the high- (Nc-Spain7) and the low-virulence (Nc-Spain1H) isolates showed marked differences in proliferation (Jiménez-Pelayo *et al*., 2017).

However, the interactions between the parasite and the placental target cells from an immunological point of view have not been investigated *in vitro* until now. Thus, the objective of the present study was to compare the immune response profiles of the bovine placental cells *in vitro* after the infection with two *N. caninum* isolates of different virulence. Messenger RNA expression levels of TLR-2, pro-inflammatory cytokines IL-8, IL-12p40, IL-17, IFN-γ, TNF-α, anti-inflammatory/regulatory cytokines TGF-β1, IL-4, IL-6 and IL-10 as well as ICAM-1 and VCAM-1 endothelial adhesion molecules were determined at 4 and 24 hours post-infection (hpi) in maternal caruncular (BCEC-1) and foetal trophoblast (F3) cell cultures and protein secretion was assessed in culture supernatants by ELISA.

2. Methods

2.1 Parasites and cell cultures

A full description of the Nc-Spain1H and Nc-Spain7 parasites and cell cultures of bovine caruncular epithelial (BCEC-1) and bovine trophoblast cells (F3) is provided in a previous report (Jiménez-Pelayo *et al*., 2017). Briefly, Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy, congenitally infected calves (Regidor-Cerrillo *et al*., 2008; Rojo-Montejo *et al*., 2009a) and tachyzoites were maintained in a MARC-145 culture as described previously (Regidor-Cerrillo *et al*., 2011). The number of culture passages of both *N. caninum* isolates was limited (passages from 9 to 11) to maintain their biological *in vivo* behavior (Pérez-Zaballos *et al*., 2005).

The BCEC-1 and F3 cell lines were kindly donated by Dr C. Pfarrer from the University of Veterinary Medicine Hannover and maintained following the protocols described in the literature (Bridger *et al*., 2007b; Hambruch *et al*., 2010).

2.2 Infection of the cultures, collection and preservation of the samples

BCEC-1 and F3 cells were seeded in 25 cm^2 culture flasks adjusting the number of cells in order to obtain a confluent monolayer after 24 h of culture. F3 was seeded at 10^6 cells per flask, whereas BCEC-1 was seeded at a concentration of 2×10^6 cells per flask. Tachyzoites were recovered from MARC-145 cultures when most of the parasites were still inside parasitophorous vacuoles; tachyzoites were purified using disposable PD-10 Desalting Columns (G.E. Healthcare, Amersham, UK) as previously described (Regidor-Cerrillo *et al*., 2011). The parasite viability was checked by trypan blue exclusion, and the tachyzoites were counted. Multiplicity of infection (MOI) of 8 (8×10^6 tachyzoites in F3 and 16×10^6 tachyzoites in BCEC-1) and 10 (10^7tachyzoites in F3 and 2×10^7 tachyzoites in BCEC-1) from the Nc-Spain7 and Nc-Spain1H isolates, respectively, were inoculated into confluent monolayers of F3 and BCEC-1 quickly after collection. Due to the differences observed in the infection rate between isolates (Jiménez-Pelayo *et al*., 2017), different MOIs of each isolate were selected with the aim of obtaining cultures infected with the same quantity of each parasite at 4 and 24 hpi. This way possible differences in the modulation of the mRNA expression levels between isolates could be attributed to differences in their biological behavior and not to the differences in the parasite burden. In addition, cultures were infected with high doses of both parasites to get a high infection of the cultures at 4 and 24 hpi so that the RNA from uninfected cells did not mask possible differences in RNA expression levels induced by the infection. The flasks were incubated at 37 °C until collection of the samples. The supernatants were collected at different time points (4, 8, 24 and 56 hpi) and stored at −80 °C for the detection of proteins by ELISA. The cultures were harvested at 4 or 24 hpi by scraping, centrifugation at 1350×*g* for 15 min at 4 °C and resuspending the pellet in 300 µl of RNAlater® (Qiagen, Hilden, Germany). The samples were stored at −80 °C prior to RNA extraction.

Two independent experiments were carried out and four replicates were obtained in each experiment.

2.3 RNA extraction, reverse transcription and quantitative real-time PCR

The mRNA expression levels of TLR-2, pro-inflammatory cytokines IL-6, IL-8, IL-12p40, IL-17, IFN-γ, TNF-α, anti-inflammatory/regulatory cytokines TGF-β1, IL-4 and IL-10 as well as ICAM-1 and VCAM-1 endothelial adhesion molecules were determined by real-time RT-PCR in the F3 and BCEC-1 cell layers infected with the high-virulence isolate (Nc-Spain7) and the low-virulence isolate (Nc-Spain1H) of *N. caninum* at an early (4 hpi) and a late (24 hpi) time point.

RNA was extracted using a commercial Maxwell® 16 LEV simplyRNA Purification kit (Promega, Madison, WI, USA) following the manufacturer's recommendations. RNA integrity was checked by 1% agarose gel and RNA concentrations were determined using a NanoPhotometer® spectrophotometer (Implen, Munich, Germany). cDNA was obtained by reverse transcription of 2.5 µg of RNA using the master mix SuperScript® VILO™ cDNA Synthesis kit (Invitrogen, Paisley, UK), which was diluted 1:20 in molecular grade water for the qPCR assays.

The PCRs were performed using 12.5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 pmol of each primer (except for TLR-2 primers which were used at a concentration of 22.5 pmol) and 5 µl of diluted cDNA samples in an ABI 7300 Real Time PCR System (Applied Biosystems). The primers used for the qPCR reactions are shown in Table 1. β-Actin and GAPDH were used as housekeeping genes, obtaining comparable Ct values for all the samples. For each target gene, a seven-point standard curve was included in each batch of amplifications based on 10-fold serial dilutions starting at 10 ng/µl of plasmid DNA. The relative quantification of the mRNA expression levels (x-fold change in expression) was carried out by the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008).

2.4 Measurement of cytokines in supernatants of BCEC-1 and F3 cell cultures by ELISA

Protein concentrations of the cytokines that showed variations in the mRNA expression levels were determined in the culture supernatants at 4, 8, 24 and 56 hpi using commercial ELISA kits. The levels of IL-6, IL-8 and TNF-α cytokines were measured in the supernatants of the BCEC-1 and F3 cells by sandwich ELISAs using a Bovine IL-6 ELISA Reagent kit (ESS0029; Thermo Fisher Scientific, Waltham, MA, USA), Bovine IL-8 (CXCL8) ELISA Development kit (3114-1A-6; Mabtech AB, Stockholm, Sweden) and Bovine TNF-α ELISA kit (EBTNF; Thermo Fisher Scientific) following the manufacturers' instructions. The sensitivity limits of these assays were 78 pg/ml for IL-6, 25 pg/ml for IL-8 and 100 pg/ml for TNF-α.

2.5 Statistical analysis

TLR, cytokine and endothelial adhesion molecule mRNA expression levels, as well as differences in the protein secretion between infected and control groups, were analysed using the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparison test for all pairwise comparisons. In addition, to assess differences between both infected groups a Mann–Whitney test was performed for each molecule analysed. The statistical significance for all the analyses was established with $P<0.05$. GraphPad Prism v.5.01 software (GraphPad Software, San Diego, CA, USA) was used to perform all statistical analyses and create all the graphical illustrations.

Table 1. Sequences of primers used for cytokine real-time PCR (qPCR) and standard curve data

Target[a]	Primer	Primer sequences (5'-3')	Product size (bp)	R^2 [b]	Slope[c]
IFN-γ (NM_174086.1)	QIFN-UP[g]	GATTCAAATTCCGGTGGATG	110	0.994	(-3.47) – (-3.30)
	QIFN-RP[g]	TTCTCTTCCGCTTTCTGAGG			
TNF-α (EU276079.1)	QTNF-UP[g]	CCAGAGGGAAGAGCAGTCC	126	0.998	(-3.39) – (-3.27)
	QTNF-RP[g]	GGAGAGTTGATGTCGGCTAC			
IL-4 (M77120.1)	QIL4-UP[g]	CTGCCCCAAAGAACACAACT	169	0.995	(-3.33) – (-3.54)
	QIL4-RP[g]	GTGCTCGTCTTGGCTTCATT			
IL-6 (X68723.1)	QIL-6-UP[d]	CTGGGTTCAATCAGGCGATT	150	0.999	(-3.22) – (-3.20)
	QIL-6-RP[d]	GGATCTGGATCAGTGTTCTGA			
IL-8 (BC103310.1)	qIL8-Fw[h]	CCACACCTTTCCACCCCAAA	177	0.995	(-3.36) – (-3.23)
	qIL8-Rw[h]	CTTGCTTCTCAGCTCTCTTC			
IL-10 (NM_174088.1)	QIL10-UP[g]	TGCTGGATGACTTTAAGGGTTACC	60	0.999	(-3.27) – (-3.42)
	QIL10-RP[g]	AAAACTGGATCATTTCCGACAAG			
IL-12p40 (NM_174356.1)	QIL12-UP[g]	AGTACACAGTGGAGTGTCAG	157	0.992	(-3.39) – (-3.35)
	QIL12-RP[g]	TTCTTGGGTGGGTCTGGTTT			
IL-17 (NM_001008412.1)	qIL17bov-up[h]	GAACTTCATCTATGTCACTGC	83	0.997	(-3.30) – (-3.18)
	qIL17bov-rev[h]	TGGACTCTGTGGGATGATGA			
TGF-β1 (NM_001009400.1)	QTGF-UP[d]	GGTGGAATACGGCAACAAAA	117	0.999	(-3.60) – (-3.53)
	QTGF-RP[d]	CGAGAGAGCAACACAGGTTC			
TLR-2 (NM_001048231.1)	QTLR2-UP[e]	ACGACGCCTTTGTGTCCTAC	192	0.993	(-3.74) – (-3.38)
	QTLR2-RP[e]	CCGAAAGCACAAAGATGGTT			
ICAM-1 (NM_174348.2)	qICAM-Fw[h]	AGACCTATGTCCTGCCATCG	219	0.994	(-3.34) – (-3.30)
	qICAM-Rw[h]	GGTGCCCTCCTCATTTTCCT			
VCAM (XM_005204079.2)	qVCAM-Fw[h]	GAACTGGAAGTCTACATCTC	128	0.998	(-3.36) – (-3.32)
	qVCAM-Rw[h]	CAGAGAATCCGTGGAGCTGG			
GAPDH (NM_001034034)	GAPDH-F[f]	ATCTCGCTCCTGGAAGATG	227	0.996	(-3.67) – (-3.58)
	GAPDH-R[f]	TCGGAGTGAACGGATTCG			
β- actin (NM_173979.3)	BACTIN-UP[g]	ACACCGCAACCAGTTCGCCAT	216	0.994	(-3.45) – (-3.36)
	BACT216-RP[g]	GTCAGGATGCCTCTCTTGCT			

[a]NCBI accession numbers are for cDNA sequences used in primer design. Primer annealing was also checked with the *Bos taurus* genomic DNA sequences (http://www.ncbi.nlm.nih.gov/nuccore)
[b]Minimum coefficient of regression (R^2) of standard curves for each PCR target in all batches of amplification
[c]Standard curve slopes. Minimum and maximum values for slopes for each PCR target in all batches of amplification
[d]Primer first described by Arraz-Solís *et al*. (Arranz-Solís *et al*., 2016)
[e]Primer first described by Menzies & Ingham (Menzies & Ingham, 2006)
[f]Primer first described by Puech *et al*. (Puech *et al*., 2015)
[g]Primer first described by Regidor-Cerrillo *et al*. (Regidor-Cerrillo *et al*., 2014)
[h]Primer described in the present work for the first time

3. Results

3.1 Expression profile of TLR-2

Our results showed that *N. caninum* infection for 4 h in BCEC-1 cells resulted in a significant upregulation of TLR-2 expression in Nc-Spain1H-infected cells compared with that of negative control cells (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2=16.2$, $df=2$, $P=0.0001$) and with that of BCEC-1 cells infected with the high-virulence isolate Nc-Spain7 (Mann–Whitney U-test: $U_{(8)}=8$, $Z=2.591$, $P=0.0007$). In F3 cultures, statistical significance was not found at either 4 or 24 hpi between infected groups and the control group. However, comparing both isolates, lower expression of TLR-2 was found in the F3

cultures infected with Nc-Spain7 than in the F3 cultures infected with the low-virulence isolate Nc-Spain1H at 4 hpi (Mann–Whitney U-test: $U_{(8)}=16$, $Z=2.287$, $P=0.0315$) (Fig. 1a).

3.2 Pro-inflammatory and regulatory cytokine modulation

The pro-inflammatory cytokines IL-8 (Kruskal–Wallis H-test: $\chi^2=19.52$, $df=2$, $P<0.0001$ in BCEC-1 and $\chi^2=17.56$, $df=2$, $P=0.0002$ in F3) and TNF-α (Kruskal–Wallis H-test: $\chi^2=19.73$, $df=2$, $P<0.0001$ in BCEC-1 and $\chi^2=19.4$, $df=2$, $P<0.0001$ in F3) were upregulated in both cell types at 4 hpi compared to the respective control groups (Fig. 1b, c). At 24 hpi, IL-8 expression was still increased in BCEC-1 cells infected by both isolates (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2=16.63$, $df=2$, $P=0.0003$ and $\chi^2=10.19$, $df=2$, $P=0.0117$ for Nc-Spain7 and Nc-Spain1H, respectively); however, the increment of IL-8 had disappeared at 24 hpi in F3-infected cells with respect to the control group. When both isolates were compared, Nc-Spain1H induced a higher expression of TNF-α than the high-virulence isolate Nc-Spain7 at 4 hpi in F3 cells (Mann–Whitney U-test: $U_{(8)}=0$, $Z=2.579$, $P<0.0001$). Protein levels of the pro-inflammatory cytokines IL-8 and TNF-α were also investigated in the supernatant of control and infected cultures at different time-points post-infection. A higher secretion of IL-8 was found for both isolates in BCEC-1 cells at 24 hpi (Kruskal–Wallis H-test: $\chi^2=15.87$, $df=2$, $P=0.0004$) and in F3 cells at 56 hpi (Kruskal–Wallis H-test: $\chi^2=13.74$, $df=2$, $P=0.001$) with respect to the control group (Fig. 2a, b). Secretion of TNF-α was higher in BCEC-1 cells infected by both isolates (Kruskal–Wallis H-test: $\chi^2=18.9$, $df=2$, $P<0.0001$; Fig. 2c) and in F3 cells infected by Nc-Spain1H (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2=16$, $df=2$, $P<0.0001$) at 8 hpi, although an earlier secretion of TNF-α was also found in F3 cells infected by Nc-Spain1H ($\chi^2=14$, $df=2$, $P=0.0003$) at 4 hpi (Fig. 2d, e). As observed with the TNF-α mRNA expression, Nc-Spain1H induced a higher secretion of TNF-α than did the high-virulence isolate Nc-Spain7 at 4 hpi (Mann–Whitney U-test: $U_{(8)}=0$, $Z=2.736$, $P=0.0002$) and at 8 hpi ($U_{(8)}=0$, $Z=2.305$, $P=0.0002$) in the F3 cultures (Fig. 2d, e).

The expression levels of other important cytokines associated with *N. caninum* infection, such as IL-12p40 and IL-6 (Fig. 1d, e), were modified in placental cells after parasite infection. Specifically, IL-6 levels were downregulated in BCEC-1 infected by Nc-Spain1H and Nc-Spain7 at 4 hpi (Kruskal–Wallis H-test: $\chi^2=16.08$, $df=2$, $P=0.0003$) and F3 cultures infected by Nc-Spain1H at 24 hpi ($\chi^2=10.5$, $df=2$, $P=0.0052$). IL-12p40 was also downregulated but only in infected F3 cultures at 4 hpi ($\chi^2=12.99$, $df=2$, $P=0.0015$). Differences between isolates in the modulation of IL-6 and IL-12p40 were not found. In addition, we observed that the caruncular cell layer did not express IL-12p40 mRNA at any time point. The decrease in the expression of IL-6 observed in infected BCEC-1 cells was confirmed by the decrease in the secretion levels of that protein found in the supernatants from BCEC-1 cultures infected with both isolates at 4 hpi, although statistically significant differences were not found (Kruskal–Wallis H-test: $\chi^2=2.765$, $df=2$, $P=0.251$), probably because of the high deviation between samples (Fig. 2f). Finally, pro-inflammatory IL-17 and IFN-γ responses were not detected in any cell lines at 4 nor at 24 hpi.

We also studied the mRNA levels of the anti-inflammatory cytokines TGF-β1, IL-4 and IL-10. Remarkably, we observed a decrease in the expression levels of TGF-β1 in both cell lines infected with both isolates. Specifically, a decrease was observed at 4 hpi in F3 cultures (Kruskal–Wallis H-test: $\chi^2=18.44$, $df=2$, $P<0.0001$) and at 24 hpi in BCEC-1 cultures ($\chi^2=12.02$, $df=2$, $P=0.0025$) (Fig. 1f). No differences between isolates were observed in the mRNA expression levels of TGF-β1. There was not detection in bovine placental cells of the anti-inflammatory cytokine IL-4 or the regulatory cytokine IL-10 at 4 and 24 hpi.

3.3 Endothelial adhesion molecule (ICAM-1 and VCAM-1) expression

The adhesion molecule ICAM-1 was expressed by both cell lines at 4 and 24 hpi. However, only a slight decrease in the mRNA expression levels of ICAM-1 was observed in the BCEC-1 cultures infected with Nc-Spain7 at 24 hpi compared to the control group although statistical significance was not found (Kruskal–Wallis H-test: $\chi^2=5.894$, $df=2$, $P=0.0525$) (Fig. 1g). VCAM-1 expression was detected only in the F3 cultures at 24 hpi, but differences between the infected and the control groups were not found in this culture at this time point (Fig. 1h).

Results of mRNA expression levels and protein secretion from statistical tests are reported in Additional file 1: Tables S1 and S2, respectively.

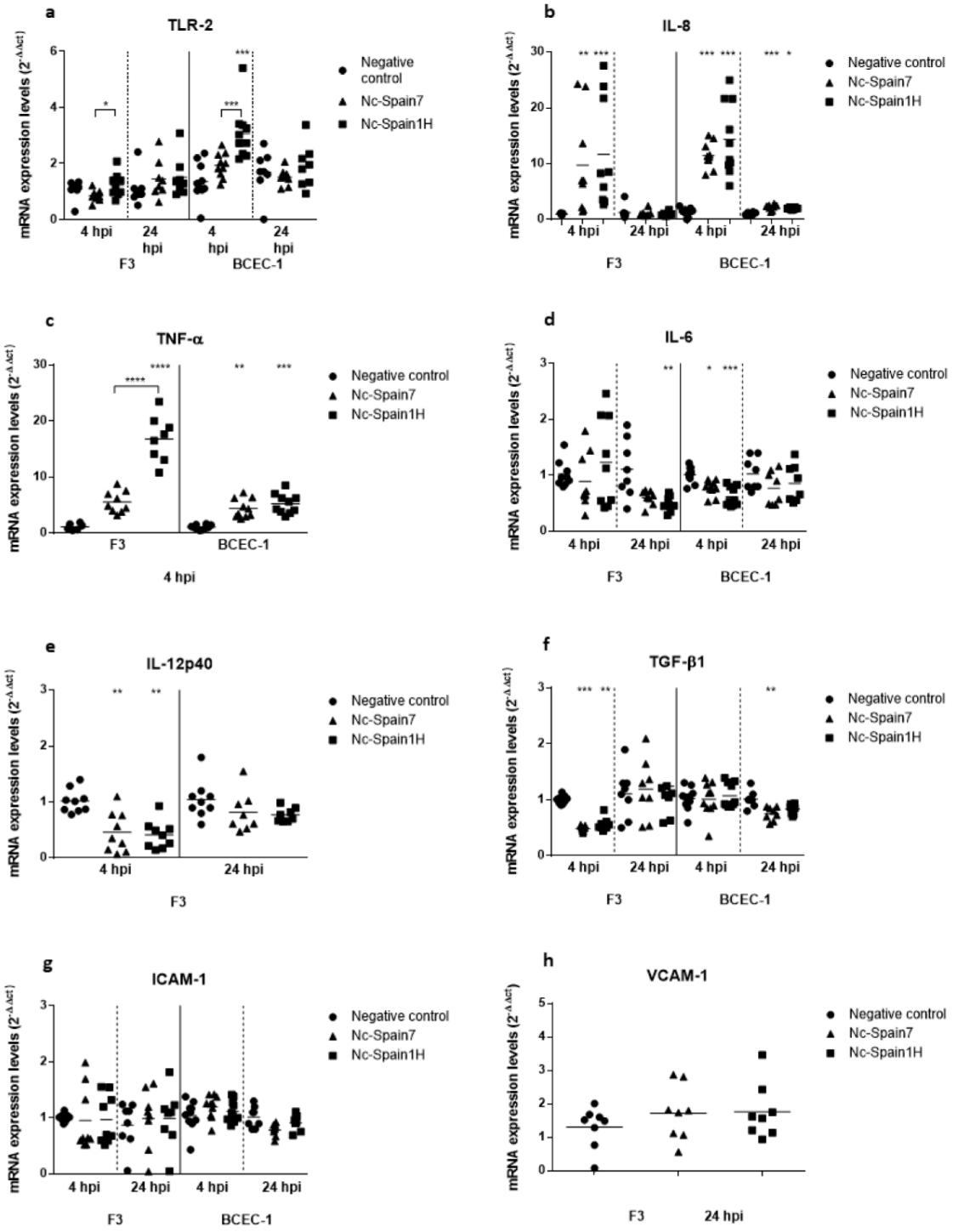

Figure 1. TLR-2, IL-8, TNF-α, IL-6, IL-12p40, TGF-β1, ICAM-1 and VCAM-1 transcript expression. Scatter-plot graphs of relative mRNA expression levels (as x-fold change) of TLR-2 (**a**), IL-8 (**b**), TNF-α (**c**), IL-6 (**d**), IL-12p40 (**e**), TGF-β1 (**f**), ICAM-1 (**g**) and VCAM-1 (**h**) in F3 and BCEC-1 cell cultures at 4 and 24 hpi with Nc-Spain7 and Nc-Spain1H isolates. Data are represented as individual points. Horizontal lines represent median values for each group. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. Unbracketed symbols represent differences with respect to the control group, while significant differences between isolates are denoted by horizontal square brackets

4. Discussion

Transmission of *N. caninum* across the placenta makes this organ key in the pathogenesis of bovine neosporosis. Innate immune signalling is crucial at the maternal-foetal interface, where vertical transmission of pathogens to the foetus can have profound pathological outcomes. Trophoblasts and other cell types within the placenta may also be involved in the physiological protection of the placenta (Bevilacqua *et al*., 2010).

Trophoblast cells have been shown to respond to some infections by producing pro-inflammatory cytokines and chemokines and endometrial or decidual cells can produce and secrete a variety of cytokines, participating in the attraction and activation of immune effector cells (Mitsunari *et al*., 2006; Gillaux *et al*., 2011). However, these innate immune mechanisms are unexplored at the maternal-foetal interface during *N. caninum* infection in pregnant cattle (Marin *et al*., 2007). The expression of TLRs has been described in trophoblasts and other cell types within the placenta (Koga & Mor, 2008). Specifically, TLR-2 was overexpressed in bovine trophoblast cell cultures at 8 hpi (Horcajo *et al*., 2017) and TLR-3, 7 and 8 have been implicated in *N. caninum* recognition in the bovine placenta (Marin *et al*., 2017a; Marin *et al*., 2017b). In our study, differential activation of TLR-2 in the F3 and BCEC-1 cultures was observed.

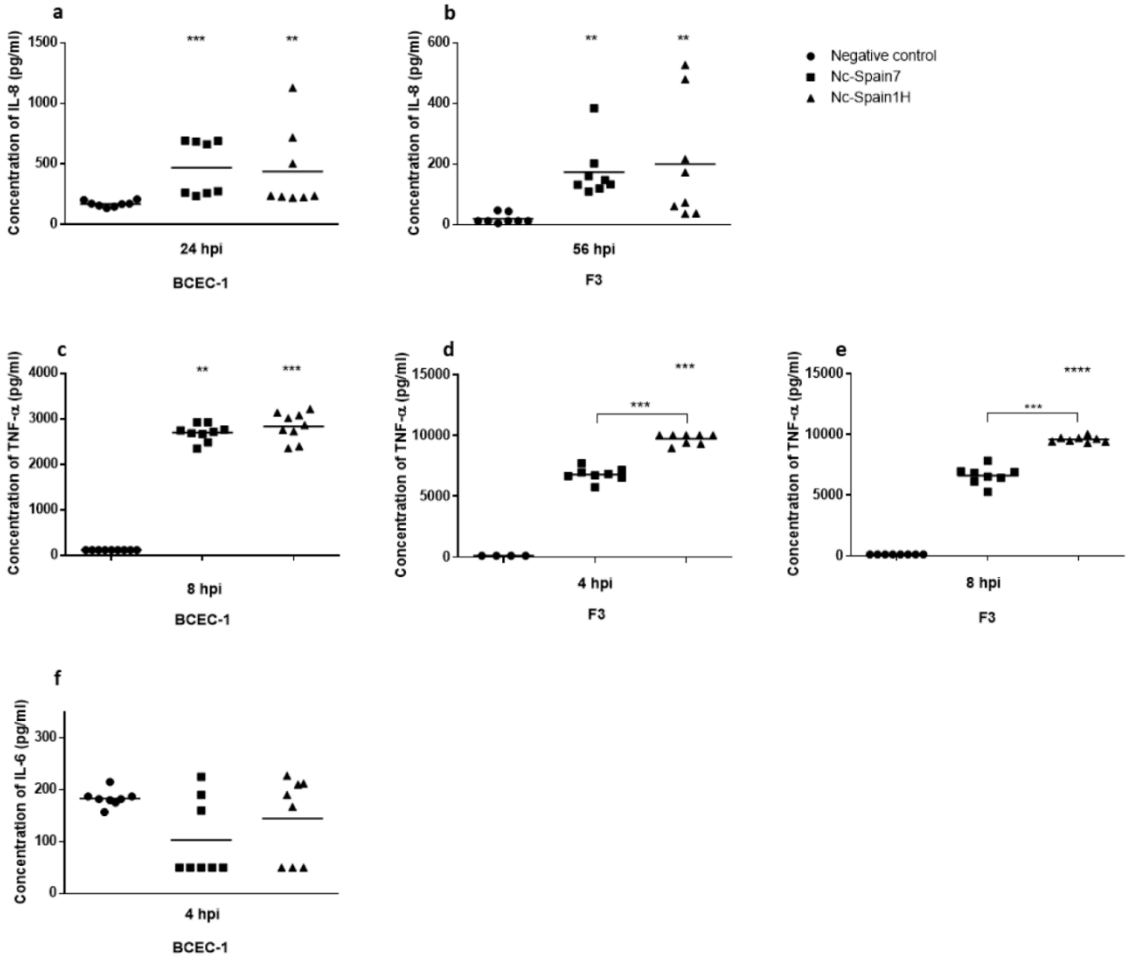

Figure 2. IL-8, TNF-α and IL-6 secretion levels in culture supernatants. Scatter-plot graphs representing the concentration of IL-8 (pg/ml) in BCEC-1 (**a**) and F3 (**b**) supernatants infected with Nc-Spain7 and Nc-Spain1H at 24 and 56 hpi, respectively, the concentration of TNF-α (pg/ml) in the BCEC-1 supernatants at 8 hpi (**c**) and in the F3 supernatants at 4 hpi (**d**) and 8 hpi (**e**), and the concentration of IL-6 (pg/ml) in the BCEC-1 supernatants at 4 hpi (**f**). Data are represented as individual points. Horizontal lines represent median values for each group. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05. Unbracketed symbols represent differences with respect to the control group, while significant differences between isolates are denoted by horizontal square brackets

An upregulation of TLR-2 was found in BCEC-1-infected cultures, especially in those infected with the low-virulence isolate Nc-Spain1H. The caruncular part of the placentome showed a higher expression of several TLRs, suggesting that the initial recognition of *N. caninum* at the placental level would occur in the maternal side of placenta (Marin *et al*., 2017a). Taking into account the data shown in Jiménez-Pelayo *et al.* (2017), which confirm the higher proliferation of *N. caninum* in trophoblast cells, an important role of placental TLR-2 in the immune response against *N. caninum* seems plausible. TLR activation is crucial for initiating the innate immune responses responsible for the elimination of intracellular parasites such as *N. caninum*, and the signalling pathway activated by TLR-2 leads to an increase in the transcription factors NF-κβ and AP-1, which trigger the synthesis of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and IL-1β) and chemokines (IL-8, RANTES) (Liu & Cao, 2016). Despite differential modulation of host TLR-2, both cell types presented a similar variation in the IL-6, TNF-α and IL-8 expression levels in infected cultures. The pro-inflammatory IL-8 and TNF-α cytokines were upregulated, and secretion of the proteins in the supernatants of both cell lines was also detected by ELISA. IL-8, a cytokine with neutrophil chemotactic/activating and T-cell chemotactic activity both *in vivo* and *in vitro*, is important in the recruitment of leukocytes to the endometrium and may be a potential mediator of placental macrophage infiltration (Adams & Rlloyd, 1997), which might help to eliminate the parasite. IL-8 upregulation has already been observed in bovine umbilical vein endothelial cells (BUVECs) infected by *T. gondii* and *N. caninum* (Taubert *et al*., 2006) as well as in bovine trophoblastic cells and placentomes from cows infected with *Brucella abortus* (Carvalho *et al*., 2008). TNF-α is an inflammatory cytokine whose expression has also been described for epithelial cells (Haider & Knöfler, 2009). TNF-α is expressed in all cell types of the trophoblastic lineage and provokes a variety of biological effects on placental and endometrial cell types (Haider & Knöfler, 2009). In addition, TNF-α, through the NF-κβ signalling pathway, coordinates the inflammatory response via the induction of other cytokines (IL-1 and IL-6) and chemokines (IL-8) and via the upregulation of adhesion molecules (ICAM-1 and VCAM-1) (Haraldsen *et al*., 1996; Cavalcanti *et al*., 2012), playing a role favouring protective immunity in infectious diseases (Robbins *et al*., 2012). There are several lines of experimental evidence indicating that TNF-α plays a role not only in immunity to *N. caninum* but also in the immunopathology of neosporosis. TNF-α expression and secretion may reduce the parasite presence in the placenta by inhibiting the intracellular multiplication of the parasite (Yamane *et al*., 2000) and participating in parasite proliferation control mechanisms (Jesus *et al*., 2013); however, TNF-α expression is detrimental to pregnancy maintenance (Rosbottom *et al*., 2008).

IL-6 expression levels were diminished in infected cultures of BCEC-1 and F3 at 4 and 24 hpi, respectively. The classification of IL-6 as Th1 or Th2 has been considered controversial since it can have characteristics of both depending on the dose, the cellular source and the gestational stage studied (Jauniaux *et al*., 1996). Currently, the presence of IL-6 displaces the Th1/Th2 balance towards a Th2 response (Diehl & Rincón, 2002). However, *in vivo* models of *N. caninum* infection have shown IL-6 upregulation (Pinheiro *et al*., 2010; Almería *et al*., 2011; Jiménez-Pelayo *et al*. unpublished data). This response pattern may be related to a protective action that protects the foetus and allows gestation even if the animals are born infected (Innes *et al*., 2002). The decrease in IL-6 observed could be explained by the following: (i) IL-6 expression levels were affected by the high antigenic dose administered (MOI 10), resulting in downregulation (Liao *et al*., 2018); (ii) the time points were not adequate for detecting the peak of IL-6 expression, and the observed decrease may be the consequence of the rapid reduction in IL-6 expression after a peak of expression (Gayle *et al*., 2004; Ashdown *et al*., 2006; Beloosesky *et al*., 2006); or (iii) other cell types are implicated in the upregulation of IL-6 that was observed *in vivo*.

The anti-inflammatory cytokine TGF-β1 was also found to be downregulated in F3 and BCEC-1 cultures at 4 and 24 hpi, respectively. Several members of the TGF-β superfamily have been suggested to regulate trophoblast cell functions, and their dysregulation has been implicated in pregnancy-associated diseases. TGF-β1 is crucial in neutralizing the inflammatory responses induced by Th1-type cytokines (Entrican, 2002). This effect has already been observed in previous works where the reduction of TGF-β1 was shown to be beneficial for controlling *N. caninum* growth but detrimental for the adequate maintenance of pregnancy (Almería *et al*., 2011; Arranz-Solís *et al*., 2016).

The reduction of pro-inflammatory IL-12p40 observed in trophoblast cells, together with the lack of expression of IL-12p40 in BCEC-1 cultures, disagrees with the results of previous experimental infections (Rosbottom *et al*., 2008; Rosbottom *et al*., 2011; Almería *et al*., 2011; Regidor-Cerrillo *et al*., 2014; Almería *et al*., 2016). Similarly, the lack of expression of pro-inflammatory IFN-γ, essential for controlling parasite infection (Baszler *et al*., 1999;

Innes *et al*., 2005); and anti-inflammatory IL-4 and IL-10, related to placental protection during *N. caninum* infections (Rosbottom *et al*., 2008; Regidor-Cerrillo *et al*., 2014), lead us to hypothesize that the upregulation of IL-12p40, IFN-γ, IL-4 and IL-10 observed *in vivo* could be attributed to immune cells present in the placenta, such as dendritic cells, NK cells or macrophages. Therefore, trophoblast and/or caruncular cells would not be responsible for the direct production of these cytokines, although the assayed time points may not have been appropriate for their detection.

Finally, ICAM-1 and VCAM-1 expression were not modulated by the parasite infection. These adhesion molecules participate in the recruitment of inflammatory immune cells (Etienne-Manneville *et al*., 1999) and promote the adherence of monocytes to endothelial cells (Deisher *et al*., 1993). The upregulation of ICAM-1 has been observed in *in vitro* infections with apicomplexan parasites (Taubert *et al*., 2006; Silva *et al*., 2015; Maksimov *et al*., 2016). The absence of modulation observed in this work may be explained by differences in the timing of the expression of ICAM-1 and VCAM-1 (Taubert *et al*., 2006; Silva *et al*., 2015; Maksimov *et al*., 2016) or by the lack of stimuli such as the acute-phase protein C-reactive protein (CRP), which is produced by the liver in response to IL-6 (Zhang *et al*., 2008).

As mentioned above, the parasite isolate is a key factor in the outcome of the infection. In general, differences in the modulation between high- and low-virulence isolates were not remarkable in trophoblast or caruncular cells, with the exception of the mRNA expression levels of TLR-2 and TNF-α. TLR-2 levels were more upregulated by Nc-Spain1H infection than by Nc-Spain7 infection in both cell lines, which led us to hypothesize that the high-virulence isolate would activate less of the TLR recognition system, reducing the immune responses triggered by TLR-2. The inhibition of the TLRs implicated in the recognition of *Trypanosoma cruzi* and *T. gondii* in HPCVE increased the parasite burden and, importantly, TLR-2 inhibition prevented the secretion of IL-6 and IL-10, increasing parasite damage (Castillo *et al*., 2017a; Castillo *et al*., 2017b). The low-virulence isolate Nc-Spain1H activates the expression of TLR-2, starting an inflammatory response, which may be the cause of the lower proliferation of this isolate (Regidor-Cerrillo *et al*., 2011; Jiménez-Pelayo *et al*., 2017), in addition to being one of the causes that explains the higher levels of TNF-α in Nc-Spain1H-infected cells, especially in trophoblast cells. Our results suggest that differential activation of the TLRs by the isolates of differing virulences should be subject to future research since they may be responsible for the biological differences observed both *in vitro* and *in vivo*.

The low-virulence isolate Nc-Spain1H also induced higher expression of TNF-α in F3. A higher TNF-α response may more efficiently control the proliferation of Nc-Spain1H in F3 cultures, which could explain the observations made by Jiménez-Pelayo *et al*. (2017) where lower proliferation of Nc-Spain1H was observed in these cells. The lower expression of TNF-α observed during the early stage of infection of trophoblasts with the high-virulence isolate Nc-Spain7 supports the hypothesis that this isolate may modify by yet unknown mechanisms the pro-inflammatory response by trophoblast cells. However, how Nc-Spain7 is able to evade the immune response and maintain lower levels of TNF-α expression in F3 remains unknown. On the other hand, these results suggest that pro-inflammatory cytokines such as TNF-α could have a minor impact in placental damage than postulated in previous works (Rosbottom *et al*., 2008; Almería *et al*., 2017), but other mechanisms should be implicated in placental damage *in vivo* and the occurrence of abortion, such as the high multiplication ability showed by the high-virulence isolate Nc-Spain7 (Jiménez-Pelayo *et al*., 2017).

5. Conclusions

The results presented in this manuscript suggest that placental cells participate in the innate immune response at the maternal-foetal interface via a rapid pro-inflammatory response characterized by the overexpression of IL-8 and TNF-α and the downregulation of TGF-β1 and IL-6. Slight differences were detected when the immunomodulatory response induced by the high and low virulent *N. caninum* isolates was compared. The higher expression of TLR-2 in the F3 and BCEC-1 cells and the TNF-α in F3 cells infected with the low-virulence isolate Nc-Spain1H may indicate a higher stimulation of the immune response by this isolate or a higher immunomodulation of Nc-Spain7, which could explain the biological differences observed *in vitro* and *in vivo*. F3 and BCEC-1 cultures seem to be a good tool for the study of the TLR activation mechanisms by *N. caninum*. Finally, we observed that cytokines such as IFN-γ, IL-4 or IL-10, which are commonly upregulated in the placenta after *N. caninum* infection, are not expressed in F3 and BCEC-1 cells; we conclude that the trophoblast and caruncular epithelial cells are not implicated in the production of these cytokines in the placenta or that other pathways/cells/molecules are needed for their production.

6. References

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Not applicable.

Authors' contributions
JRC, PH, ECF, MGB and LMO conceived the study and participated in its design. LJP and MGS wrote the manuscript, with interpretation of results and discussion input from JRC, ECF, MGB, LMO, NH and CP. LJP and MGS performed *in vitro* infection of the cultures, collection of the samples and ELISA assays. JRC, PH, LJP and MGS designed and performed RT-

qPCR analyses. NH and CP isolated bovine trophoblast and caruncular cell lines used in the assays. LJP and MGS carried out statistical analyses and interpreted the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Additional file 1: Table S1. Statistical test results for mRNA expression levels.

mRNA expression levels	BCEC-1							
	4 hpi				24 hpi			
	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)
ICAM-1	$\chi^2 = 2.341$, $df = 2$, $P = 0.3103$	$\chi^2 = 5.9$, $df = 2$, $P = 0.4019$	$\chi^2 = 1.9$, $df = 2$, $P > 0.9999$	$U_{(8)} = 37$, $Z = 1.016$, $P = 0.3527$	$\chi^2 = 5.894$, $df = 2$, $P = 0.0525$	$\chi^2 = 8.188$, $df = 2$, $P = 0.0517$	$\chi^2 = 2.688$, $df = 2$, $P > 0.9999$	$U_{(8)} = 15.5$, $Z = 1.6$, $P = 0.0768$
IL-12p40	$\chi^2 = 0.3789$, $df = 2$, $P = 0.8386$	$\chi^2 = 2$, $df = 2$, $P > 0.9999$	$\chi^2 = 1$, $df = 2$, $P > 0.9999$	$U_{(8)} = 20$, $Z = 0.3194$, $P = 0.9452$	—	—	—	—
IL-6	$\chi^2 = 16.08$, $df = 2$, $P = 0.0003$	$\chi^2 = 9.9$, $df = 2$, $P = 0.0357$	$\chi^2 = 15.6$, $df = 2$, $P = 0.0002$	$U_{(8)} = 24$, $Z = 1.448$, $P = 0.0524$	$\chi^2 = 2.822$, $df = 2$, $P = 0.2439$	$\chi^2 = 5.813$, $df = 2$, $P = 0.2928$	$\chi^2 = 3.75$, $df = 2$, $P = 0.8554$	$U_{(8)} = 25$, $Z = 0.5879$, $P = 0.4828$
IL-8	$\chi^2 = 19.52$, $df = 2$, $P < 0.0001$	$\chi^2 = 14.2$, $df = 2$, $P = 0.0009$	$\chi^2 = 15.8$, $df = 2$, $P = 0.0002$	$U_{(8)} = 42$, $Z = 0.4064$, $P = 0.5787$	$\chi^2 = 16.13$, $df = 2$, $P = 0.0003$	$\chi^2 = 16.63$, $df = 2$, $P = 0.0003$	$\chi^2 = 10.19$, $df = 2$, $P = 0.0117$	$U_{(8)} = 18$, $Z = 0.9742$, $P = 0.1506$
TFG-β1	$\chi^2 = 0.09548$, $df = 2$, $P = 0.9534$	$\chi^2 = 0.1$, $df = 2$, $P > 0.9999$	$\chi^2 = 1$, $df = 2$, $P > 0.9999$	$U_{(8)} = 44$, $Z = 0.2794$, $P = 0.6842$	$\chi^2 = 12.02$, $df = 2$, $P = 0.0025$	$\chi^2 = 11.75$, $df = 2$, $P = 0.002$	$\chi^2 = 7.75$, $df = 2$, $P = 0.0735$	$U_{(8)} = 18$, $Z = 1.161$, $P = 0.1602$
TLR-2	$\chi^2 = 17.31$, $df = 2$, $P = 0.0002$	$\chi^2 = 6$, $df = 2$, $P = 0.3825$	$\chi^2 = 16.2$, $df = 2$, $P = 0.0001$	$U_{(8)} = 8$, $Z = 2.591$, $P = 0.0007$	$\chi^2 = 1.915$, $df = 2$, $P = 0.3838$	$\chi^2 = 4.063$, $df = 2$, $P = 0.7482$	$\chi^2 = 0.3125$, $df = 2$, $P > 0.9999$	$U_{(8)} = 22$, $Z = 1.24$, $P = 0.3138$
TNF-α	$\chi^2 = 19.73$, $df = 2$, $P < 0.0001$	$\chi^2 = 13.8$, $df = 2$, $P = 0.0014$	$\chi^2 = 16.2$, $df = 2$, $P = 0.0001$	$U_{(8)} = 38$, $Z = 0.6096$, $P = 0.393$	—	—	—	—
VCAM-1	—	—	—	—	—	—	—	—

Abbreviations: NC: negative control, *vs*: *versus*

Additional file 1: Table S1. Continued.

mRNA expression levels	F3							
	4 hpi				24 hpi			
	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)
ICAM-1	χ^2 = 1.03, *df* = 2, *P* = 0.5975	χ^2 = 3.778, *df* = 2, *P* = 0.938	χ^2 = 2.222, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 38, *Z* = 0.4157, *P* = 0.8633	χ^2 = 0.6865, *df* = 2, *P* = 0.7094	χ^2 = 2.438, *df* = 2, *P* > 0.999	χ^2 = 2.625, *df* = 2, *P* > 0.999	$U_{(8)}$ = 32, *Z* = 0.05304, *P* > 0.9999
IL-12p40	χ^2 = 12.99, *df* = 2, *P* = 0.0015	χ^2 = 11.33, *df* = 2, *P* = 0.0074	χ^2 = 12, *df* = 2, *P* = 0.004	$U_{(8)}$ = 39, *Z* = 0.1782, *P* = 0.9314	χ^2 = 4.655, *df* = 2, *P* = 0.0975	χ^2 = 7, *df* = 2, *P* = 0.1431	χ^2 = 6.125, *df* = 2, *P* = 0.2496	$U_{(8)}$ = 27, *Z* = 0.2475, *P* = 0.6454
IL-6	χ^2 = 1.115, *df* = 2, *P* = 0.5727	χ^2 = 3.778, *df* = 2, *P* = 0.938	χ^2 = 0.8889, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 36, *Z* = 0.7721, *P* = 0.7304	χ^2 = 10.5, *df* = 2, *P* = 0.0052	χ^2 = 7.438, *df* = 2, *P* = 0.1004	χ^2 = 11.13, *df* = 2, *P* = 0.0044	$U_{(8)}$ = 18.5, *Z* = 1.054, *P* = 0.1800
IL-8	χ^2 = 17.56, *df* = 2, *P* = 0.0002	χ^2 = 17.67, *df* = 2, *P* = 0.0021	χ^2 = 14.33, *df* = 2, *P* = 0.0004	$U_{(8)}$ = 33, *Z* = 0.4454, *P* = 0.5457	χ^2 = 1.097, *df* = 2, *P* = 0.5778	χ^2 = 0.375, *df* = 2, *P* > 0.9999	χ^2 = 3, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 24, *Z* = 0.9554, *P* = 0.4258
TFG-β1	χ^2 = 18.44, *df* = 2, *P* < 0.0001	χ^2 = 15.44, *df* = 2, *P* = 0.0001	χ^2 = 11.56, *df* = 2, *P* = 0.006	$U_{(8)}$ = 23, *Z* = 1.039, *P* = 0.1359	χ^2 = 0.05446, *df* = 2, *P* = 0.9731	χ^2 = 0.3125, *df* = 2, *P* > 0.9999	χ^2 = 0.5, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 31, *Z* = 0.2313, *P* = 0.9361
TLR-2	χ^2 = 6.628, *df* = 2, *P* = 0.0364	χ^2 = 8.111, *df* = 2, *P* = 0.0905	χ^2 = 0.4444, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 16, *Z* = 2.287 *P* = 0.0315	χ^2 = 4.511, *df* = 2, *P* = 0.1048	χ^2 = 6.375, *df* = 2, *P* = 0.2099	χ^2 = 6.563, *df* = 2, *P* = 0.1863	$U_{(8)}$ = 31.5, *Z* = 0.0533, *P* = 0.9807
TNF-α	χ^2 = 19.4, *df* = 2, *P* < 0.0001	χ^2 = 7.5, *df* = 2, *P* = 0.1077	χ^2 = 16, *df* = 2, *P* < 0.0001	$U_{(8)}$ = 0, *Z* = 2.579, *P* < 0.0001	—	—	—	—
VCAM-1	—	—	—	—	χ^2 = 1.235, *df* = 2, *P* = 0.5393	χ^2 = 3.625, *df* = 2, *P* = 0.9157	χ^2 = 3.125, *df* = 2, *P* > 0.9999	$U_{(8)}$ = 31, *Z* = 0.1414, *P* = 0.9591

Additional file 1: Table S2. Statistical results for protein secretion.

Protein secretion		BCEC-1				F3			
Cytokine	Time	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)	NC *vs* Nc-Spain7 *vs* Nc-Spain1H (Kruskal-Wallis H-test)	NC *vs* Nc-Spain7 (Dunn's multiple comparison test)	NC *vs* Nc-Spain1H (Dunn's multiple comparison test)	Nc-Spain7 *vs* Nc-Spain1H (Mann-Whitney U-test)
IL-6	4 hpi	$\chi^2 = 2.765$, $df = 2$, $P = 0.251$	$\chi^2 = 5.5$, $df = 2$, $P = 0.3383$	$\chi^2 = 1.25$, $df = 2$, $P > 0.9999$	$U_{(8)} = 22$, $Z = 1.225$, $P = 0.2886$	—	—	—	—
	24 hpi	$\chi^2 = 15.87$, $df = 2$, $P = 0.0004$	$\chi^2 = 13.25$, $df = 2$, $P = 0.0005$	$\chi^2 = 10.75$, $df = 2$, $P = 0.0071$	$U_{(8)} = 22$, $Z = 0.7073$, $P = 0.3282$	—	—	—	—
IL-8	56 hpi	—	—	—	—	$\chi^2 = 13.74$, $df = 2$, $P = 0.001$	$\chi^2 = 11.88$, $df = 2$, $P = 0.0022$	$\chi^2 = 10.63$, $df = 2$, $P = 0.0076$	$U_{(8)} = 29$, $Z = 0.3551$, $P = 0.7984$
	4 hpi	—	—	—	—	$\chi^2 = 16.84$, $df = 2$, $P < 0.0001$	$\chi^2 = 6$, $df = 2$, $P = 0.2817$	$\chi^2 = 14$, $df = 2$, $P = 0.0003$	$U_{(8)} = 0$, $Z = 2.736$, $P = 0.0002$
TNF-α	8 hpi	$\chi^2 = 18.9$, $df = 2$, $P < 0.0001$	$\chi^2 = 11.78$, $df = 2$, $P = 0.004$	$\chi^2 = 15.22$, $df = 2$, $P = 0.0001$	$U_{(8)} = 25$, $Z = 0.9379$, $P = 0.1903$	$\chi^2 = 21.26$, $df = 2$, $P < 0.0001$	$\chi^2 = 8$, $df = 2$, $P = 0.0635$	$\chi^2 = 16$, $df = 2$, $P < 0.0001$	$U_{(8)} = 0$, $Z = 2.305$, $P = 0.0002$

Abbreviations: NC: negative control, *vs*: *versus*

Objetivo 2

Investigación de la dinámica de infección temprana en un modelo bovino gestante inoculado con aislados de alta y baja virulencia de *N. caninum* en el segundo tercio de la gestación.

La neosporosis bovina es una de las causas más importantes de aborto en ganado bovino en todo el mundo, apareciendo con mayor frecuencia durante la mitad de la gestación en condiciones naturales. Además, la variabilidad biológica de *N. caninum* ha sido ampliamente descrita anteriormente, sin embargo, las causas que la producen no han sido descubiertas todavía. Con el objetivo de confirmar los hallazgos hechos en los ensayos *in vitro* en células placentarias bovinas de carúncula y cotiledón se implementó un modelo bovino gestante infectado a la mitad de gestación, en el que se compararon la dinámica de la infección así como la respuesta inmunitaria local en la placenta y la modulación de la matriz extracelular (MEC) inducida por dos aislados de distinta virulencia de *N. caninum*, Nc-Spain7, de alta virulencia, y Nc-Spain1H, de baja virulencia, en dos momentos tempranos tras la infección (10 y 20 días post-infección, dpi). Veinticuatro novillas de la raza Asturiana fueron separadas aleatoriamente en tres grupos: el grupo G-Control, formado por 6 animales, fue inoculado con PBS, el grupo G-NcSpain7 formado por 9 animales fue inoculado por vía intravenosa con 10^7 taquizoítos del aislado más virulento Nc-Spain7, y el grupo G-NcSpain1H, formado por 9 animales, fue inoculado por vía intravenosa con 10^7 taquizoítos del aislado menos virulento Nc-Spain1H. Tres animales del G-Control y 4 animales de cada grupo infectado fueron sacrificados en el día 10 pi mientras que el resto de animales fueron sacrificados a los 20 dpi. Las consecuencias clínicas, la distribución del parásito en los tejidos maternos, fetales y placentarios así como el desarrollo de lesiones fue comparado entre los dos aislados a los 10 y 20 dpi. Tras la inoculación con ambos aislados se detectó un pico de fiebre en el día 1 pi, que se repitió en el día 3 pi sólo en el grupo infectado con el aislado más virulento. A los 10 dpi, se detectó ADN del parásito en muestras de placenta de un animal infectado con el aislado Nc-Spain7, asociado a la aparición de necrosis focal. Además, se observó transmisión de este aislado a los tejidos fetales, aunque en este caso no se asoció con presencia de lesiones. Sin embargo, el aislado menos virulento no fue detectado a los 10 dpi en tejidos maternos, fetales ni placentarios, y tampoco se observaron lesiones asociadas a la presencia del parásito. A los 20 dpi, prácticamente el 100% de las muestras de placenta fueron positivas a la presencia de parásito en los animales infectados con el aislado Nc-Spain7, asociado a necrosis focalizada severa. Además, la transmisión al feto se produjo en todos los animales de este grupo, hallándose mortalidad fetal en dos de ellos. Sin embargo, el aislado menos virulento sólo fue detectado en muestras placentarias de un animal a los 20 dpi, que no fueron asociadas a lesiones. Tampoco se detectó transmisión ni lesiones en órganos fetales. En cuanto a la respuesta inmunitaria periférica, todos los animales infectados con Nc-Spain7 habían presentado anticuerpos específicos frente a *N. caninum* a los 20 dpi mientras que sólo 3 de los 5 animales infectados con Nc-Spain1H habían seroconvertido. Además, dicha seroconversión fue observada antes en los animales infectados con el aislado más virulento. En resumen, los taquizoítos del aislado más virulento, Nc-Spain7, alcanzaron antes la placenta, multiplicándose y dando lugar al desarrollo de lesiones, transmisión

y muerte fetal mientras que el aislado menos virulento mostró una infección más tardía de la placenta sin desarrollo de lesiones ni transmisión durante los primeros 20 días tras la infección. Todos estos hallazgos parecen estar relacionados con una mejor capacidad del aislado más virulento para invadir las células de la placenta, así como una mayor capacidad de proliferación, como se demostró *in vitro*.

Por otro lado, las muestras de placenta, tanto materna como fetal, obtenidas en este experimento fueron analizadas por técnicas de inmunohistoquímica y PCR cuantitativa con el objetivo de comparar la respuesta inmunitaria local en la placenta inducida por aislados de distinta virulencia. Además, distintos componentes de la matriz extracelular (MEC) fueron también investigados, ya que la organización de dicha matriz ha sido indicada como uno de los principales puntos de modulación por la infección del parásito y se ha postulado como un mecanismo que podría facilitar el paso de barreras biológicas como la placenta. En general, una respuesta caracterizada por un aumento tanto de las citoquinas Th1 como Th2 (principalmente IFN-γ e IL-4) junto con una respuesta inflamatoria caracterizada por un infiltrado mayoritariamente de linfocitos T CD4+ y CD8+ fueron observadas en todas las muestras infectadas, aunque el incremento tanto de citoquinas como de células inflamatorias fue más marcado a los 20 dpi. Además, se detectaron diferencias interesantes entre ambos aislados. A los 10 dpi se observó un aumento generalizado de la expresión de todas las citoquinas únicamente en los animales infectados con el aislado menos virulento, desencadenando una respuesta inmunitaria frente a este aislado que puede ayudar a controlar la infección. Sin embargo, el reconocimiento del aislado más virulento, Nc-Spain7, no se produjo hasta los 20 dpi, lo que podría contribuir a su multiplicación y diseminación. La detección tardía del parásito, cuando las lesiones ya se han desarrollado, podría dar lugar a una respuesta inmunitaria exacerbada en la placenta, en un intento del hospedador por controlar la infección, que, a su vez, podría contribuir a la muerte fetal. Específicamente, mayores niveles de IL-8, TNF-α e iNOS, mayor infiltración de células inmunitarias y menor expresión del TGF-β1 fueron detectados en las placentas de los animales que presentaron muerte fetal en comparación con aquellos animales infectados con el mismo aislado que no presentaron muerte fetal a los 20 dpi, sugiriendo que éstos pueden estar directamente involucrados en el aborto asociado a *N. caninum*. Además, una profunda alteración de la MEC, con disminución de la expresión de MMP-2 y TIMP-2 y destrucción de colágeno, fibronectina y vimentina, y la falta de activación de procesos de reparación tisular podrían contribuir al mecanismo de aborto. Finalmente, la expresión de los componentes de la MEC se encontró aumentada en los animales infectados con el aislado menos virulento, Nc-Spain1H, tanto a los 10 como a los 20 dpi, demostrando que dicho aislado es capaz de inducir la remodelación tisular que, por un lado, podría contribuir a la migración de células inmunitarias periféricas al foco de la infección pero, por otro lado, podría contribuir también a la transmisión del parásito al feto. En resumen, se observaron diferentes patrones de interacción parásito-hospedador a nivel de la placenta bovina durante la infección temprana a mitad de gestación con aislados de alta y baja virulencia de *N. caninum*, lo que sugiere la existencia de diferentes estrategias adaptativas evolutivas utilizadas por este parásito para la transmisión a la descendencia.

Sub-objective 2.1: Early *N. caninum* infection dynamics in pregnant heifers after inoculation at mid gestation with high- and low-virulence isolates.

Jiménez-Pelayo L[1], García-Sánchez M[1], Vázquez P[1], Regidor-Cerrillo J[2], Horcajo P[1], Collantes-Fernández E[1], Blanco-Murcia J[3], Gutiérrez-Expósito D[4], Román-Trufero A[5], Osoro K[5], Benavides J[4], Ortega-Mora LM[1]*.

[1]Saluvet, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[2]Saluvet-innova, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[3]Department of Animal Medicine and Surgery, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[4]Instituto de Ganadería de Montaña (CSIC-Universidad de León), 24346, León, Spain.
[5]Regional Service for Research and Agri-Food Development (SERIDA), 33300 Villaviciosa, Asturias, Spain
*Corresponding author: Luis Miguel Ortega-Mora

Running title: Early *Neospora caninum* infection dynamics in cattle after inoculation at mid-gestation with high (Nc-Spain7)- or low (Nc-Spain1H)-virulence isolates

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Abstract

Early *Neospora caninum* infection dynamics were investigated in pregnant heifers intravenously inoculated with PBS (G-Control) or 10^7 tachyzoites of high (G-NcSpain7)- or low- (G-NcSpain1H) virulence isolates at 110 days of gestation. Serial culling at 10 and 20 days post-infection (dpi) was performed. Fever was detected at 1 dpi in both infected groups ($P < 0.0001$), and a second peak was detected at 3 dpi only in G-NcSpain7 ($P < 0.0001$). At 10 dpi, Nc-Spain7 was detected in placental samples from one animal related to focal necrosis, and Nc-Spain7 transmission was observed, although no foetal lesions were associated with this finding. The presence of Nc-Spain1H in the placenta or foetuses, as well as lesions, were not detected at 10 dpi. At 20 dpi, G-NcSpain7 animals showed almost 100% positive placental tissues and severe focal necrosis as well as 100% transmission. Remarkably, foetal mortality was detected in two G-NcSpain7 heifers. Only one animal from G-NcSpain1H presented positive placental samples. No foetal mortality was detected, and lesions and parasite transmission to the foetus were not observed in this group. Finally, 100% of G-NcSpain7 heifers at 20 dpi presented specific antibodies, while only 60% of G-NcSpain1H animals presented specific antibodies at 20 dpi. In addition, earlier seroconversion in G-Nc-Spain7 was observed. In conclusion, tachyzoites from Nc-Spain7 reached the placenta earlier and multiplied, leading to lesion development, transmission to the foetus and foetal mortality, whereas Nc-Spain1H showed delayed infection of the placenta and no lesional development or transmission during early infection.

Keywords

Neospora caninum, pregnant cattle, mid-gestation, Nc-Spain7, Nc-Spain1H, early infection, virulence

1. Introduction

Neospora caninum is an apicomplexan protozoan parasite that is considered to be one of the main causes of abortion in cattle. Horizontal transmission via oocyst ingestion is possible, although transplacental transmission in cattle seems to be the most efficient infection route (Williams *et al*., 2009). In pregnant cattle, infection with this parasite may lead to abortion, birth of still-born calves, birth of new-born calves with clinical signs or birth of clinically healthy but persistently infected calves (Dubey *et al*., 2007; Dubey & Schares, 2011). The disease outcome is influenced by several factors, including the maternal immune response in the placenta and the relative immunocompetence of the foetus at the time of infection, which are two key variables (Benavides *et al*., 2014; Horcajo *et al*., 2016). Experimental *N. caninum* infection in pregnant cattle during the first term generally produces foetal death and abortion, and foetuses show more severe lesions (Williams *et al*., 2000; Regidor-Cerrillo *et al*., 2014). Experimental infection from the second trimester onward, which is when the foetal immune system begins to develop, generally results in clinically healthy but congenitally infected calves (Williams *et al*., 2000; Gibney *et al*., 2008; Almería *et al*., 2010; Benavides *et al*., 2012), although infection with the highly virulent isolate Nc-Spain7 induced at least 50% foetal death at 110 days of gestation (dg) (Almería *et al*., 2016, Vázquez *et al*., submitted). Under natural conditions, abortion caused by *N. caninum* is more frequent during the second trimester of pregnancy (Collantes-Fernández *et al*., 2006a; Almería & López-Gatius, 2013).

A limited number of studies have been conducted to investigate the consequences of *N. caninum* infection at mid-gestation (Maley *et al*., 2003; Bartley *et al*., 2004; Almería *et al*., 2010; Almería *et al*., 2016; Vázquez *et al*., submitted). Recently, intravenous (IV) inoculation of 10^7 tachyzoites of Nc-Spain7 at mid-gestation produced 50% foetal death until 42 days post-infection (dpi) (Almería *et al*., 2016) and 66.6% foetal death when gestation lasted until term. Moreover, foetal death was

observed using lower doses of Nc-Spain7 tachyzoites, although a lower percentage of abortions and a delayed presentation were detected as the dose decreased (Vázquez *et al*., submitted).

The outcome of the infection in pregnant cattle also depends on the isolate. Specifically, the high-virulence isolate Nc-Spain7 showed a percentage of abortion and vertical transmission of 100% in a bovine model at early gestation (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014), whereas the infection in experimentally infected cattle with the low-virulence isolate Nc-Spain1H did not result in foetal death (Rojo-Montejo *et al*., 2009b).

In the current study, the aim was to investigate how the differences between high (Nc-Spain7)- and low(Nc-Spain1H)- virulence isolates of *N. caninum* influence the clinical outcome, parasite distribution and burden, lesion development in placental and foetal tissues, and the specific antibody response during early infection in pregnant heifers inoculated at mid-gestation. The lack of bovine models studying early infection and the lack of experimental infections comparing isolates make the implementation of this model necessary to elucidate the pathogenesis of bovine neosporosis at mid-gestation, which is when most abortions occur in naturally infected cattle (Anderson *et al*., 1995; López-Gatius *et al*., 2004).

2. Materials and methods

2.1 Animals and experimental design

Asturiana heifers, aged 20-30 months, were selected after assessing their seronegativity to *N. caninum*, Infectious Bovine Rhinotracheitis (IBR) virus, Bovine Viral Diarrhoea (BVD) virus, Leptospira and Mycobacterium avium subsp. paratuberculosis by ELISA. The health and reproductive management of the animals is detailed in Additional File 1. Pregnant heifers (n=24) were randomly distributed into three experimental groups, G-Control (n=6), G-NcSpain7 (n=9) and G-NcSpain1H (n=9) and inoculated intravenously at 110 days of gestation with phosphate buffered saline (PBS) and 107 culture-derived tachyzoites of Nc-Spain7 and Nc-Spain1H isolates, respectively. Three animals from G-Control, four animals from G-NcSpain7 and four animals from G-NcSpain1H were culled at 10 dpi, while three animals from G-Control, five animals from G-NcSpain7 and five animals from G-NcSpain1H were culled at 20 dpi.

2.2 Parasites

Nc-Spain7 and Nc-Spain1H tachyzoites were routinely maintained in cultured MARC-145 cells, and inoculum was prepared as described previously (Regidor-Cerrillo *et al*., 2010). The same limited parasite passage numbers for both isolates were used for the experimental infection (11) to ensure the maintenance of their *in vivo* biological characteristics and avoid adaptation to the host cell (Pérez-Zaballos *et al*., 2005). Briefly, tachyzoites were recovered from culture flasks when they were still largely intracellular, and at least 80% of the parasitophorous vacuoles were undisrupted. Tachyzoite numbers were determined by Trypan blue exclusion followed by counting in a Neubauer chamber, and parasites were resuspended in PBS at the required dose of 107 tachyzoites in a final volume of 2 ml. Tachyzoites were administered to heifers within 1 h of harvesting from tissue culture.

2.3 Clinical monitoring and sampling

Cattle were observed daily before and after inoculation throughout the entire experimental period. Rectal temperatures were recorded daily from 6 days prior to challenge to 14 dpi and weekly from 14 dpi onward. Animals with temperatures above 39.5 ºC were considered to be febrile. Foetal viability was checked once a week by ultrasound scanning of foetal heartbeat and movements. Blood samples were collected by coccygeal venipuncture at days -6 and -1 and twice a week until the end of the experiment for further analyses.

Animals were sedated with xylazine hydrochloride (Rompun; Bayer, Mannheim, Germany) and euthanised by an IV overdose of embutramide and mebezonio iodide (T61; Intervet, Salamanca, Spain). Post-mortem examination of the heifers was performed immediately after euthanasia. Foetuses were separated from the placenta, and 18 placentomes (6 cranial, medial and caudal) were randomly recovered from each placenta. Half of the placentomes from each area were carefully detached by hand, and maternal caruncles (CA) and foetal cotyledons (CO) were separated. Full placentomes were transversally cut in slices measuring 2-3 mm in thickness, which were distributed for storage in 10% formalin (Sigma-Aldrich, Saint Louis, MO, USA) for histopathological examinations. Both full placentomes and CA and CO sections were also stored at -80ºC for parasite DNA detection by PCR. Foetal tissues included the brain (FB), heart, liver (FL), lung and a portion of semitendinosus skeletal muscle, which were maintained at -80 ºC for DNA extraction and fixated in 10% formalin. Blood and foetal thoracic and abdominal fluids were also collected when possible and maintained at -80ºC for serological analysis. Heifer tissues, including pre-scapular and ileofemoral lymph nodes, were also collected for PCR and histopathological analysis.

2.4 Histopathology and lesion quantification

After fixation for two weeks, maternal and foetal samples and placentomes were trimmed and conventionally processed for embedding in paraffin wax and haematoxylin and eosin (HE) staining. Histological slides were studied under an optical microscope. Lesion quantification at placentome samples was performed through a computer-assisted morphometric analysis in HE stained sections following a previously described procedure (Arranz-Solis *et al*., 2015). Among the parameters evaluated were the number and size of necrotic foci (NF and ASF), as well as the total area of necrotic lesions (%LES) affecting the interdigitate area of the placentome. In addition, the accumulation of proteinaceous material (eosinophilic) and cellular debris in the haemophagus subchondral area of the placentome was also measured, and the results are expressed as a ratio between the area occupied by the proteinaceous exudate in the haemophagus zone and the total area of the placentome.

2.5 Tissue DNA extraction and PCR determinations

DNA extraction and PCR determinations were carried out as described elsewhere (Regidor-Cerrillo *et al*., 2014; Arranz-Solís *et al*., 2015). Briefly, genomic DNA was extracted from 20-100 mg of maternal and foetal tissue samples using the Maxwell® 16 Mouse Tail DNA Purification Kit (Promega, Wisconsin, USA). Parasite DNA was detected by nested PCR adapted to a single tube from the internal transcribed spacer (ITS1) region of *N. caninum* using TgNN1-TgNN2 and NP1-NP-2 as external and internal primers, respectively (Buxton *et al*., 1998; Hurtado *et al*., 2001; Regidor-Cerrillo *et al*., 2014). DNA quantification was performed by real-time PCR using the equipment ABI 7500 FAST (Applied Biosystems, Foster City, CA, USA) and targeting Nc-5 as described previously (Collantes-Fernández *et al*., 2002). Detailed information concerning DNA extraction and PCR is given in Additional File 1.

2.6 IFN-γ responses in sera

IFN-γ levels in sera from dams were measured by the Bovine IFN-γ ELISA development kit (Mabtech AB, Sweden) following the manufacturer's recommendations. The colour reaction was developed by the addition of 3,3',5,5'-tetramethylbenzidine substrate (TMB, Sigma-Aldrich, Spain) and incubated for 5-10 min in the dark. Reactions were stopped by adding 2N H_2SO_4 when the first point of the standard curve reached a DO of 0.7 at 620 nm. Then, plates were read at 450 nm. The cytokine concentrations were calculated by interpolation from a standard curve generated with recombinant cytokines provided with the Bovine IFN-γ ELISA development kit (Mabtech AB, Sweden).

2.7 *N. caninum*-specific IgG responses

Neospora-specific IgG antibody levels were measured in maternal serum by ELISA (Regidor-Cerrillo *et al*., 2014). IgG1 and IgG2 subclasses were also assessed by ELISA using peroxidase-conjugated sheep anti-bovine IgG1 and IgG2 antibodies (Serotec, Oxford, UK) at 1:1000 as secondary conjugates. For each plate, the OD values were converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample - OD405 negative control)/(OD405 positive control - OD405 negative control)×100. A RIPC value ≥ 12 indicates a positive result.

Indirect fluorescent antibody test (IFAT) and Western blotting (WB) were carried out to detect specific IgG anti-*Neospora* antibodies in foetal blood and foetal thoracic and abdominal fluids. IFAT was carried out following the methodology previously described (Álvarez-García et al., 2003). Samples were diluted at two-fold serial dilutions in PBS starting at 1:8 up to the end point titre. Intact tachyzoite membrane fluorescence at a titre≥8 was considered a positive reaction. WB was carried out as described previously (Álvarez-García et al., 2002). After blocking overnight, the membranes containing tachyzoite extracts were incubated with foetal sera and fluids diluted 1:20 and incubated for 1.5 h at room temperature. After washing, the membranes were incubated with 1:1200 peroxidase-conjugated monoclonal goat anti-bovine IgG (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h, washed and developed using 4-chloro-1-naphtol (Bio-Rad Laboratories, CA, USA) as a substrate.

2.8 Statistical analysis

Differences in PCR detection of parasite DNA in maternal, foetal and placental tissues were evaluated using X2 or Fisher's exact F-test. Parasite burdens were analysed using the non-parametric Mann-Whitney U test. Occurrence of foetal death was analysed by the Kaplan-Meier survival method to estimate the percentage of viable foetuses (VF) at each time point (Bland & Altman, 1998). The foetal survival curves of the infected groups were then compared with the Gehan-Wilcoxon test. Differences in histological scoring were analysed using the non-parametric Kruskal–Wallis test followed by Dunn's test for all pairwise comparisons. Finally, a two-way ANOVA test followed by a Tukey´s multiple comparisons test, was performed to compare rectal temperatures, antibody responses and IFN-γ kinetics in sera.

Statistical significance for all analyses was established with $P < 0.05$. All statistical analyses were carried out using GraphPad Prism 5 v.5.01 software (San Diego, CA, USA)

3. Results

A summary of results (clinical outcome, lesions, parasite distribution and IgG responses) in heifers and foetuses inoculated with PBS, 107 tachyzoites of Nc-Spain7 isolate or Nc-Spain1H isolate at 110 dg and culled at 10 or at 20 dpi is shown in Table 1.

3.1 Clinical observations

The mean rectal temperatures of animals from G-Control, G-NcSpain7 and G-NcSpain1H are represented in Figure 1. Five animals from G-NcSpain7 and 5 animals from G-NcSpain1H exhibited fever at 1 dpi. Six animals from G-NcSpain7 also exhibited fever at 3 dpi, and two of these animals maintained fever until 4 dpi

The mean rectal temperatures of G-NcSpain7 increased significantly (> 39.5 ºC) at 1 and 3 dpi, and the mean rectal temperature of G-NcSpain1H only increased significantly at 1 dpi compared to the uninfected G-Control group ($P < 0.0001$; two-way ANOVA test). Significant differences between infected groups were found at 3 dpi when a second peak of fever was detected in G-NcSpain7 but not in G-NcSpain1H ($P < 0.0001$; two-way ANOVA test). Interestingly, only 5 out of 9 G-Nc-Spain1H heifers presented fever, whereas all G-Nc-Spain7 heifers were febrile at some time during the experimental period. Rectal temperatures of G-Control animals remained below 39ºC.

Foetal mortality was not detected until 20 dpi. Foetal death was detected during culling in two heifers (3581 and 7934) from G-NcSpain7 as detailed below. However, foetuses from G-Control and G-NcSpain1H remained viable throughout the experiment. The comparative analysis of foetal survival curves between infected groups showed non-significant differences ($P = 0.13$; Gehan-Wilcoxon test).

3.2 Pathology and lesion quantification

Gross lesions

Placental detachment from the uterus, and autolyzed CO, were found in the two heifers (3581 and 7934) from G-NcSpain7 that were culled at 20 dpi and presented foetal mortality. In these cases, foetuses were swollen because of subcutaneous oedema and showed a degree of autolysis. Apart from these findings, no evident gross lesions were found in the placentas, foetuses or maternal lymph nodes studied from any of the other heifers.

Microscopic lesions

Maternal lymph nodes: Histological changes were not found in any lymph node.

Placentomes: Two different histological changes were found in the placentomes.

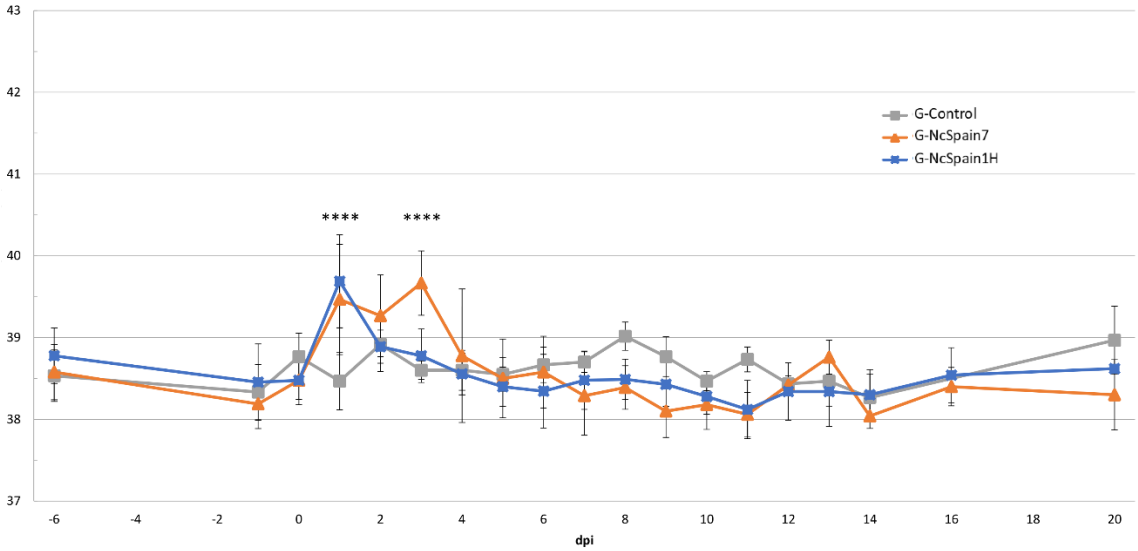
The first change consisted of focal necrosis with a variable degree of inflammatory infiltrate adjacent to the lesion, randomly distributed within the interdigitate zone of the placentome (Additional File 2a, b). This lesion was only found in G-NcSpain7 heifers, but there were differences within this group, as only one heifer culled at 10 dpi (9665) showed this lesion, affecting only one out of nine studied placentomes. The lesion was also found in all G-Nc-Spain7 animals culled at 20 dpi. All the parameters quantified in these lesions, NF, ASF and %LES, were higher in animals culled at 20 dpi than those found in the only animal with placental lesions culled at 10 dpi. Among those culled at 20 dpi, one of them (3581, non-viable foetuses -NVF-) had more NF and more %LES than the rest ($P <0.01$; Kruskal-Wallis test) (Additional file 3a, c). The ASF was higher in two animals (3581, NVF and 5082, VF) than in the other three (Additional file 3b). When studying the influence of the location of the placentomes (cranial, medial or caudal) on the evaluated parameters, no differences were found between the five heifers culled at 20 dpi. However, when analysing those two animals with higher ASF, medial and caudal CO showed higher %LES ($P <0.05$; Kruskal-Wallis test) than the cranial ones (Additional file 3c).

The second histological change found in placentomes was the accumulation of proteinaceous material (eosinophilic) and cellular debris at the haemophagus area of the placentome, i.e., extravasated plasma (Figure 2a). This accumulation was found in all the animals from the study, including G-Control. However, comparing the amount of extravasated plasma, measured as the relative area occupied by the proteinaceous material in the haemophagus area, there were clear differences between groups (Figure 2b). Placentomes from G-Control showed less accumulation than G-NcSpain7 ($P <0.0001$; Kruskal-Wallis test) and G-NcSpain1H ($P <0.001$; Kruskal-Wallis test), but the accumulation in G-NcSpain7was higher than in G-NcSpain1H ($P <0.05$; Mann-Whitney U test). Comparing the differences within each group depending on the day of culling (10 vs 20 dpi), differences were found only at G-NcSpain7, where animals culled at 10 dpi showed less accumulation than those culled at 20 dpi.

Foetal viscera. Only the five foetuses from G-NcSpain7 heifers culled at 20 dpi showed

histological lesions. The livers of all five foetuses showed perivascular aggregation of lymphocytes, macrophages and plasma cells. NVF (3581 and 7934) also showed mild multifocal necrotic foci in the liver with scant presence of inflammatory cells related to them. In addition, all foetuses from G-NcSpain7 culled at 20 dpi showed scant, randomly distributed aggregation of mononuclear cells in the lung parenchyma. NVF (3581 and 7934) also showed similar lesions in the lungs plus perivascular infiltration of mononuclear cells and, in one of them (7934), mild multifocal necrosis. Finally, we also found mild mononuclear myositis and myocarditis (Additional File 2c) in all five foetuses. Multifocal randomly distributed small aggregation of mononuclear cells in the neuropil of the brain (Additional file 2d) was observed only in VF as the brain samples from NVF were too autolytic to allow proper histological evaluation.

a



b

	Ear tag	0 dpi	1 dpi	2 dpi	3 dpi	4 dpi	5 dpi
	1600	38,3	**39,6**	38,7	39,3	38,4	37,8
	3581	38,7	38,9	39,1	**39,9**	**39,5**	38,3
	7934	38,5	**40,2**	**40,2**	**40**	38,6	38
	9665	37,9	**39,8**	39	39,4	38,6	39,1
Nc-Spain7	**5850**	38,8	38,7	39,4	**39,8**	38,5	38,6
	9661	38,2	39,3	39	**39,7**	38,8	38,4
	7992	38,5	**40,6**	39,3	39	38	38,2
	4405	38,6	**39,5**	**39,9**	**40,3**	38	39,1
	5082	38,8	38,6	38,8	**39,6**	**40,6**	39
	9131	38,2	38,9	38,7	38,5	38,4	38,1
	7725	38,5	**39,9**	39,3	39,1	38,9	38,7
	3712	38,8	**40,2**	39	38,6	38,8	38,6
	9677	38,5	39,3	38,9	39,4	38,3	38,5
Nc-Spain1H	**5925**	38,5	39,1	38,9	38,4	38,6	38
	7649	38,5	39,3	38,6	38,6	38,5	38,3
	9638	38,8	**40,6**	39	38,6	38,9	38,6
	9671	38,1	**40,1**	38,8	38,8	38,3	38,3
	3894	38,4	**39,8**	38,8	39	38,3	38,5

Figure 1. Rectal temperatures. The mean rectal temperatures of animals from G-Control, G-NcSpain7 and G-NcSpain1H during the experiment are represented in the graphic **(a)**. The exact temperatures of each infected animal recorded during the first 5 dpi are represented in table **(b)**. **** indicates $P < 0.0001$ significant differences.

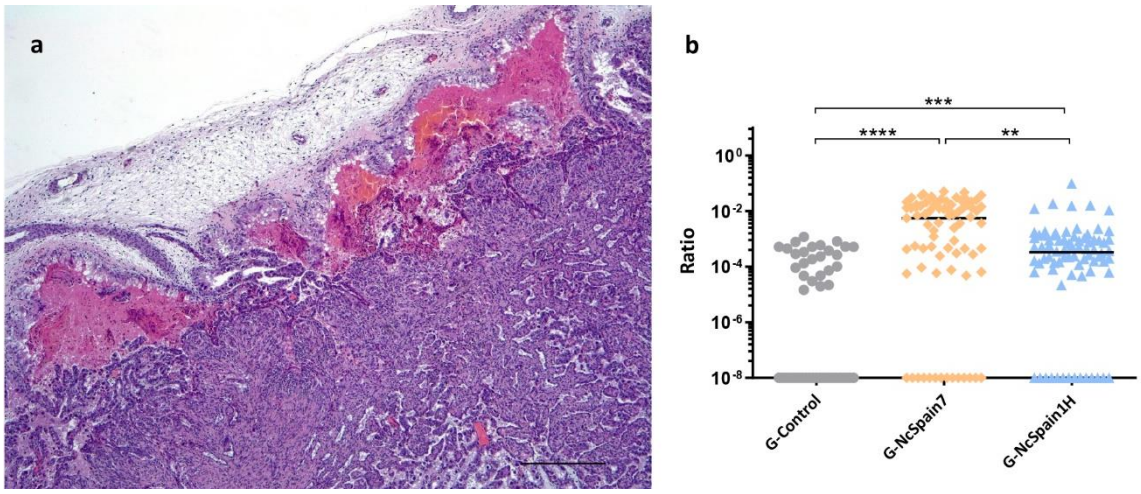


Figure 2. Proteinaceous exudate at the haemophagus area of the placentome. Representative image of accumulation of proteinaceous material and cellular debris at the haemophagus subchondral area of a placentome from G-NcSpain1H at 20 dpi. HE. 2× **(a)**, and dot-plot graph showing significant differences between groups **(b)**. Each dot represents individual values of relative area occupied by the exudate in each placentome analysed, and medians are represented as horizontal lines. ******, ***** and **** indicate $P < 0.0001$, $P < 0.001$ and $P < 0.01$ significant differences. Bar 1000 µm.

3.3 Parasite distribution and burden in placental and foetal tissues

Parasite burdens are represented in Figure 3.

Maternal tissues

N. caninum DNA was only detected in 1 out of 15 pre-scapular lymph node samples in one heifer from G-NcSpain1H culled at 20 dpi (9638).

Placental tissues

In G-NcSpain7, *Neospora* DNA was detected sporadically in CA (4/36) and CO (1/36) samples belonging to one animal culled at 10 dpi (9665) and in 44 out of 45 CA and 44 out of 45 CO samples of animals culled at 20 dpi. The differences in the frequency of parasite detection between animals from G-NcSpain7 culled at 10 and at 20 dpi were statistically significant for CA and for CO ($P < 0.0001$; Fisher exact test). In G-NcSpain1H, all CA and CO samples from animals culled at 10 dpi were negative, and only 1 out of 45 CA and 4 out of 45 CO samples from one animal culled at 20 dpi (9638) were *N. caninum* DNA positive. The frequency of detection in CA and CO in G-NcSpain7 was significantly higher than in G-NcSpain1H culled at 20 dpi ($P<0.0001$; Fisher exact test). Placental samples from G-Control animals were negative.

The parasite burden in CA and CO, measured as the number of tachyzoites per mg of tissue, was analysed in *N. caninum* DNA-positive samples. Higher parasite burdens were found in CA and CO from G-NcSpain7 at 20 dpi than in samples from G-NcSpain7 at 10 dpi ($P < 0.0001$; Mann-Whitney U test). Slightly higher parasite burdens were found in CO than in CA samples from G-NcSpain7 culled at 20 dpi, although the differences were not statistically significant ($P > 0.05$; Mann-Whitney U test) (Figure 3c). The parasite burden was higher in CA ($P \leq 0.001$; Mann-Whitney U test) and CO ($P < 0.0001$; Mann-Whitney U test) from animals with NVF (3581 and 7934) than those carrying VF (7992, 4405 and 5082) of G-NcSpain7 at 20 dpi (Figure 3d). In contrast, differences in the parasite burden in CA and CO between animals from G-NcSpain1H culled at 10 and 20 dpi were not found ($P > 0.5$; Mann-Whitney U test). Comparing animals from infected groups culled at 20 dpi, higher parasite burdens in CA and CO were detected in G-NcSpain7 than in G-NcSpain1H ($P <0.0001$; Mann-Whitney U test) (Figure 3a).

Foetal tissues

Regarding foetal tissues, 12 out of 15 FB samples from G-NcSpain7 foetuses at 20 dpi were positive by PCR, whereas all FB samples from G-NcSpain7 foetuses at 10 dpi were negative. All FBs from G-NcSpain1H foetuses culled at 10 or 20 dpi were negative. On the other hand, 2 G-NcSpain7 foetuses at 10 dpi (3/12) and one G-NcSpain7 foetus at 20 dpi (3/15) presented *N. caninum* positive FL samples, although differences between culling at 10 or 20 dpi were not found ($P = 1$; Fisher exact test). FL from all G-NcSpain1H foetuses were negative for *N. caninum* DNA detection. FB and FL samples from G-Control were negative.

Higher parasite burdens were found in FB samples from G-NcSpain7 culled at 20 dpi than at 10 dpi ($P <0.0001$; Mann-Whitney U test), but differences were not found in FL ($P > 0.5$; Mann-Whitney U test)

(Figure 3b). In addition, a higher parasite burden was found in FB than in FL in G-NcSpain7 at 20 dpi (Figure 3c). Comparing animals carrying VF and NVF from G-NcSpain7 at 20 dpi, higher parasite burdens were found in the FL of NVF ($P < 0.05$; Mann-Whitney U test), whereas no differences were found in the FB ($P > 0.5$; Mann-Whitney U test) (Figure 3d).

3.4 IFN-γ kinetics in sera

A peak of IFN-γ production was detected at 2 dpi in both infected groups with respect to the control group G-Control ($P < 0.0001$ and $P = 0.0013$ in G-NcSpain7 and G-NcSpain1H, respectively; two-way ANOVA test). All animals from G-NcSpain7 and G-NcSpain1H presented increased levels of IFN-γ at 2 dpi. Differences between infected groups were also found, with the increase of IFN-γ being higher in Nc-Spain7-infected animals than in Nc-Spain1H-infected animals ($P < 0.0002$; two-way ANOVA test) (Figure 4).

3.5 Specific anti-*Neospora* IgG responses in heifers and foetuses

N. caninum-specific antibody responses (total IgG, IgG1 and IgG2) are shown in Figure 5. An earlier detection of *N. caninum* antibodies was observed in G-NcSpain7 (9 dpi) (7934) than in G-NcSpain1H (13 dpi). All animals from G-NcSpain7 seroconverted from 13 dpi, while only 3 out of 5 animals from G-NcSpain1H seroconverted between 13 (9677) and 16 dpi (7649 and 9638).

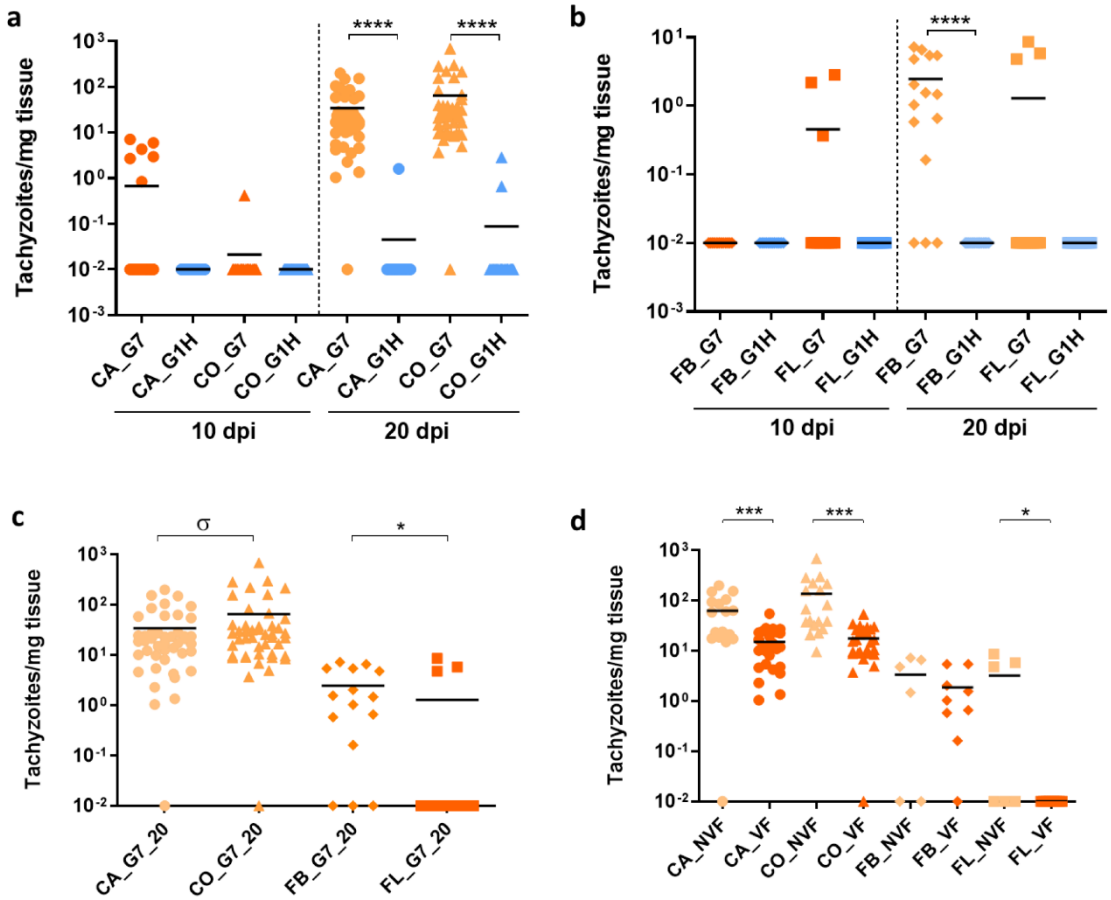


Figure 3. *N. caninum* burdens in placental and foetal tissues. Comparative of parasite burdens quantified by qPCR in nested-PCR positive samples from CA and CO **(a)** and FB and FL **(b)** from G-NcSpain7 and G-NcSpain1H heifers culled at 10 and 20 dpi. **(c)** Comparative of parasite quantification by qPCR between CA and CO and between FB and FL from G-NcSpain7 heifers culled at 20 dpi. **(d)** Comparative of parasite quantification by qPCR in samples from CA, CO, FB and FL from NVF and VF foetuses from G-NcSpain7 culled 20 dpi. Each dot represents individual values of parasite burden, and medians are represented as horizontal lines. The *N. caninum* detection limit by real-time PCR was 0.1 parasites, and negative samples (0 parasites) were represented on the log scale as<0.1 (i.e., 10^{-2}). ****, ***, and * indicate $P<0.0001$, $P<0.001$ and $P<0.05$ significant differences. *σ* indicates $P<0.1$ tendency towards significant differences.

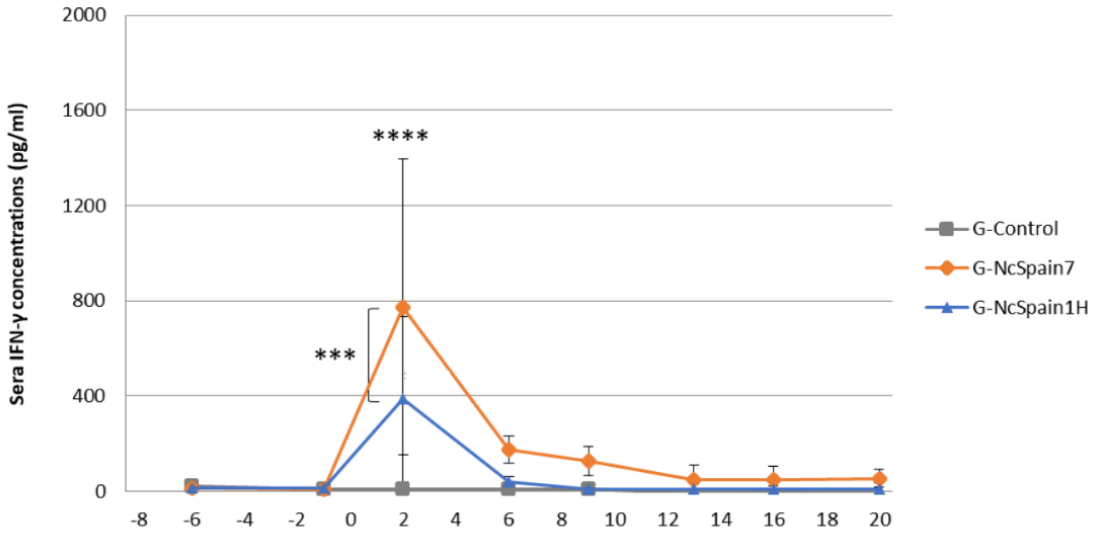


Figure 4. IFN-γ kinetics in sera. Sera concentration of IFN-γ determined by ELISA in G-Control, G-NcSpain7 and G-NcSpain1H. **** and *** indicate *P*<0.0001 and *P*<0.001 significant differences.

Total IgG levels were significantly higher from 13 dpi until the end of the experiment in G-NcSpain7 compared to G-Control ($P < 0.05$; two-way ANOVA test). No significant increase in the antibody levels was found in G-NcSpain1H during the experimental period compared to the control group ($P > 0.05$; two-way ANOVA test). Total IgG levels of G-NcSpain7 were higher than G-NcSpain1H at 16 dpi ($P < 0.05$; two-way ANOVA test) and at 20 dpi ($P < 0.0001$; two-way ANOVA test). No significant differences were found in G-NcSpain7 at 20 dpi between animals carrying NVF (3581 and 7934) and VF (7992, 4405 and 5082) (Figure 5a).

IgG1 and IgG2 kinetics were similar to those observed in total IgG levels. Higher levels of IgG1 and IgG2 were found in G-NcSpain7 than in G-Control at 16 and 20 dpi ($P < 0.01$; two-way ANOVA test). There were no significant differences in IgG1 and IgG2 levels between G-NcSpain1H and G-Control. Comparing both infected groups, higher IgG1 and IgG2 levels were found in G-NcSpain7 than in G-NcSpain1H at 16 and 20 dpi ($P < 0.01$; two-way ANOVA test), and higher IgG1 levels were also found in G-NcSpain7 than in G-NcSpain1H at 13 dpi ($P < 0.01$; two-way ANOVA test). No significant differences in IgG1 and IgG2 were found between animals carrying NVF (3581 and 7934) and VF (7992, 4405 and 5082) in G-NcSpain7 at 20 dpi (Figures 5b and 5c).

Neospora-specific IgG was not detected in foetal serum or foetal fluids by IFAT and WB.

4. Discussion

Abortion due to *N. caninum* infection appears more frequently at mid-gestation in naturally infected cattle. However, few studies of pregnant bovine models of neosporosis at this gestation time have been reported, especially those investigating early infection dynamics. In the present work comparisons between high (Nc-Spain7)- and low (Nc-Spain1H)-virulence isolates of *N. caninum* inoculated at mid-gestation were approached at early time points post-infection (10 and 20 dpi). The results from this experimental model will increase the knowledge about biological differences found between high- and low-virulence isolates *in vivo*, clarifying some of the key events involved in the pathogenesis of bovine neosporosis.

Half of the animals from both infected groups showed fever as the first clinical sign associated with *N. caninum* infection, which is in agreement with previous reports where a transient rise in body temperature was recorded during the first week post-infection, likely the consequence of the first cycle of parasite replication in host tissues (Buxton *et al*., 1998; Williams *et al*., 2000; Macaldowie *et al*., 2004; Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014; Almería *et al*., 2016; Vázquez *et al*., submitted). A second peak of fever was detected at 3 dpi only in Nc-Spain7-infected animals, which suggests an earlier and higher replication of this isolate, leading to a second antigenic exposition of Nc-Spain7 tachyzoites. Similarly, high doses of the Nc-1 isolate were associated with a bi-phasic

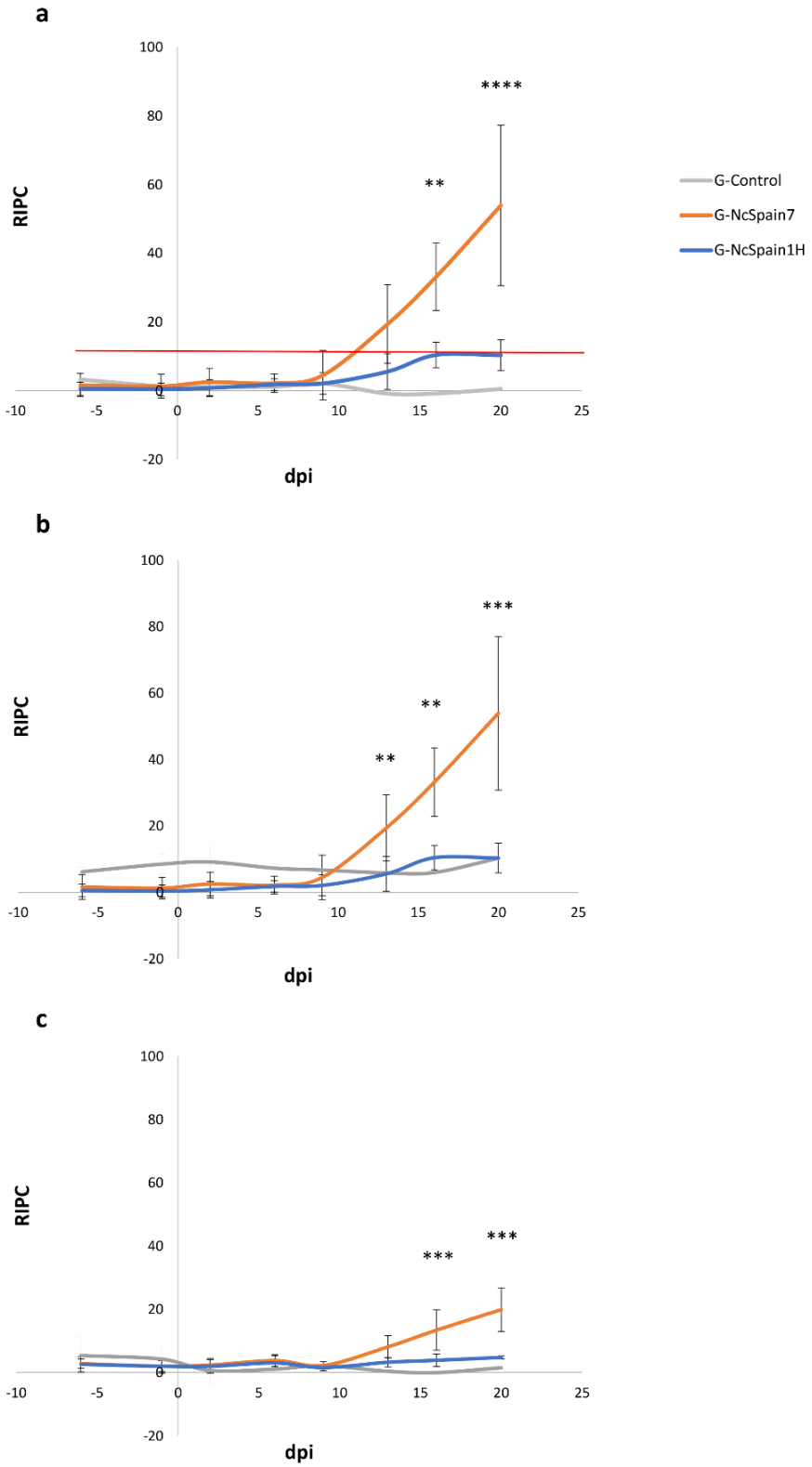


Figure 5. *N. caninum*-specific humoural immune responses. Serum levels of total IgG **(a)**, IgG1 **(b)** and IgG2 **(c)** antibodies against *N. caninum* in G-Control, G-NcSpain7 and G-NcSpain1H. Immunoglobulin levels are expressed as a relative index percent (RIPC) according to RIPC = (OD_{405} sample - OD_{405} negative control)/(OD_{405} positive control - OD_{405} negative control)×100. Each point represents the mean + SD at different sampling times. The red line indicates the ELISA cut-off point from which samples are considered positive. **** and ** indicate $P<0.0001$ and $P<0.001$ significant differences.

Table 1. Summary of early infection dynamics in heifers and foetuses from G-Control, G-NcSpain7 and G-NcSpain1H

Culling date	*Inoculum*	Ear tag	Pregnancy outcome	*Placenta*		*Foetus*			*N. caninum*-specific IgG levels in dams
				Histopathology	DNA detection	Histopathology	DNA detection	*N. caninum*-specific IgG	
10 dpi	*PBS*	5702	L	PA*	-	-	-	No	No
		1334	L	PA*	-	-	-	No	No
		6676	L	PA*	-	-	-	No	No
	Nc-Spain7	1600	L	PA**	-	-	-	No	No
		9665	L	FN*, PA**	CA ++, CO +	-	Li+	No	No
		5850	L	PA**	-	-	-	No	No
		9661	L	PA**	-	-	Li ++	No	No
	Nc-Spain1H	9131	L	PA**	-	-	-	No	No
		3712	L	PA**	-	-	-	No	No
		5925	L	PA**	-	-	-	No	No
		9671	L	PA**	-	-	-	No	No
20 dpi	*PBS*	3710	L	PA*	-	-	-	No	No
		6671	L	PA*	-	-	-	No	No
		6377	L	PA*	-	-	-	No	No
	Nc-Spain7	3581	D	FN***, PA***	CA +++, CO +++	Li**, Lu**, H, Sk, CNS†	CNS ++	No	Yes (13 dpi)
		7934	D	FN**, PA***	CA +++, CO +++	Li**, Lu***, H, Sk, CNS†	CNS ++, Li +++	No	Yes (9 dpi)
		7992	L	FN**, PA***	CA +++, CO +++	Li*, Lu*, H, Sk, CNS	CNS ++	No	Yes (13 dpi)
		4405	L	FN**, PA***	CA +++, CO +++	Li*, Lu*, H, Sk, CNS	CNS +++	No	Yes (13 dpi)
		5082	L	FN***, PA***	CA +++, CO +++	Li*, Lu*, H, Sk, CNS	CNS +++	No	Yes (16 dpi)
	Nc-Spain1H	7725	L	PA**	-	-	-	No	No
		9677	L	PA**	-	-	-	No	Yes (13 dpi)
		7649	L	PA**	-	-	-	No	Yes (16 dpi)
		9638	L	PA**	CA +, CO ++	-	-	No	Yes (16 dpi)
		3894	L	PA**	-	-	-	No	No

PA *, **, *** (protein accumulation): arbitrary degree of accumulation of sera (eosinophilic) and cellular debris at the haemophagus subchondral area of the placentome.
FN *, **, *** (focal necrosis): arbitrary degree of focal necrosis with inflammatory infiltrate in the interdigitate zone of the placentome.
Li *, ** (liver): perivascular aggregation of lymphocytes, macrophages and plasma cells and mild multifocal necrotic foci.
Lu *, **, *** (lung): aggregation of mononuclear cells in the parenchyma, perivascular mononuclear inflammation and mild multifocal necrosis
CNS†: CNS autolytic, evaluation of lesions was not possible
CA/CO +, ++, +++ (caruncle/cotyledon): 1-3 positive samples, 4-6 positive samples or 7-9 positive samples.
Li/CNS +, ++, +++ (liver/central nervous system): 1, 2 or 3 positive samples.
-: Negative/No lesion
L: Live foetus
D: Dead foetus
Sk: Skeletal muscle
H: Heart
CNS: Central nervous system

increase in rectal temperature (Maley *et al*., 2003). Moreover, all G-NcSpain7-heifers were febrile, whereas only half of G-NcSpain1H animals showed fever. Differences between isolates may be explained by a more efficient replication of Nc-Spain7, as was previously demonstrated *in vitro* (Regidor-Cerrillo *et al*., 2011; Jiménez-Pelayo *et al*., 2017; García-Sánchez *et al*., 2019) and *in vivo* (Regidor-Cerrillo *et al*., 2010).

Peripheral immune responses, both cellular and humoural, were assessed in dams' sera during the experiment. IFN-γ was detected in both infected groups at 2 dpi, demonstrating that *N. caninum* tachyzoites activated the cellular immune response, which is crucial for host defence against intracellular pathogens (Innes *et al*., 2002; Innes *et al*., 2005; Williams *et al*., 2007). Higher IFN-γ levels in G-Nc-Spain7 did not correlate with protection against infection, since the Nc-Spain7 isolate was detected earlier in the placenta and showed a more effective invasion and proliferation than the Nc-Spain1H isolate. Specific antibodies against *N. caninum* were detected in all Nc-Spain7 heifers slightly earlier than in previous works (Bartley *et al*., 2004; Almería *et al*., 2010; Almería *et al*., 2016), whereas later seroconversion, and in fewer animals, was found in three Nc-Spain1H-infected heifers. Previous reports suggested that Nc-Spain7 may be able to induce a higher antibody response, whereas antigenic stimulation seems to be more reduced in Nc-Spain1H, probably due to its lower ability to multiply in host tissues, its clearance by the immune system or both (Buxton, *et al*., 1991; Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2014).

In this study, culling as early as 10 and 20 dpi was effective in showing clear differences between isolates of variable virulence. At 10 dpi, few placental samples from one Nc-Spain7-infected animal were positive for *N. caninum,* and one of

them showed focal necrosis, demonstrating the colonization of the placenta by this isolate. Early detection of Nc-Spain7 at 10 dpi may be associated with its higher abilities for invasion and proliferation in placental cells.

To the best of our knowledge, none of the previous experiments studied the dynamics of the infection as early as 10 dpi, however similar to our observations, focal necrosis was described in placentomes at 14 dpi with the Nc-1 isolate (Maley *et al*., 2003) and at 2 wpi with the Nc-Spain7 isolate (Almería *et al*., 2016). In addition to focal necrosis, differences in the plasma extravasation were found between infected and control animals. The extravasation of erythrocytes and plasma into the haemophagus zone of the placentome is a normal finding in healthy animals (Murai & Yamauchi, 1986). However, in this study, higher extravasation was found in infected animals at 10 and 20 dpi. Previous studies have described serum leakage in relation to necrotic and inflammatory foci in the interdigitate area of the placenta (Maley *et al*., 2003; Almería *et al*., 2010), but the increase in the proteinaceous material in the haemophagus area has not been previously reported. This increase may appear as one of the initial changes in the placenta associated with *N. caninum* infection, and it is tempting to hypothesize that it might be related to changes in vascular permeability. It has been recently shown that complement and coagulation cascades were modified after *N. caninum* infection in trophoblast cells *in vitro* (Horcajo *et al*., 2017); therefore, studying early vascular events in the placenta after *N. caninum* infection could be an interesting future research. *N. caninum* was also detected in the liver of two Nc-Spain7-infected foetuses, indicating that the high-virulence isolate is already transmitted to the foetus at 10 dpi. Previous studies have suggested the crossing and presence of tachyzoites in foetal tissues as early as 10 dpi (Barr *et al*., 1994; Maley *et al*., 2003). Our findings suggest that the liver is the first target organ in the foetus, which most likely represents the gateway for the parasite to invade the foetus through the umbilical vein, replicating in the parenchyma and spreading through the foetal body, as previously observed in sheep (Arranz-Solís *et al*., 2015). However, antibodies against *N. caninum* were not found in serum or corporal fluids from any foetus, probably because antibodies cannot be produced in infected foetuses until at least 6 weeks after infection (Caspe *et al*., 2012; Almería *et al*., 2016).

In contrast, Nc-Spain1H was not found in any placental or foetal tissue, and specific humoural responses were not found either in Nc-Spain1H-infected foetuses. Lesions were not found either in placentas or in foetuses from G-NcSpain1H, apart from serum extravasation in placentomes. However, IV inoculation is supposed to disseminate the parasite quickly through the organism. We hypothesize that the presence of Nc-Spain1H in the placenta at 10 dpi is very low, since we were unable to detect the parasite, which is associated with lower invasion and replication abilities, as previously observed in placental tissues *in vitro*, especially in caruncular cells (Jiménez-Pelayo *et al*., 2017). In addition, a higher stimulation of the innate immune responses by the low-virulence isolate at the placental level was suggested at early time points post-infection *in vitro* (Jiménez-Pelayo *et al*., 2019), which could explain the more effective control of the parasite, thereby contributing to its lower proliferation.

At 20 dpi, foetal death was detected in two Nc-Spain7-infected heifers, whereas it was not detected in any Nc-Spain1H-infected heifer. All G-NcSpain7 animals presented almost 100% positive placental samples, which is in keeping with previous studies where Nc-Spain7 or other isolates showed dissemination in placental tissues early after infection (2-4 wpi) when inoculation was carried out at mid-gestation (110-140 dg) (Maley *et al*., 2003; Almería *et al*., 2010; Rojo-Montejo *et al*., 2013; Almería *et al*., 2016). Our results again demonstrated the "tropism" of *N. caninum* for the bovine placental tissue, which seems to be one of the most appropriate niches for its multiplication. Placental necrosis was observed in G-NcSpain7 animals at 20 dpi associated with high parasite burdens. The extravasation of proteinaceous material and cellular debris in the haemophagus area in G-NcSpain7 was larger at 20 dpi than at 10 dpi and larger than in G-NcSpain1H animals, suggesting a correlation between the presence and severity of this histological change and infection by *N. caninum* in the present work, since larger areas of extravasation were observed in animals with higher parasite burden and infected with more virulent isolates. Parasite was found in most FB samples from G-NcSpain7, and inflammatory infiltrate and lesions compatible with *N. caninum* were found especially in foetal CNS, which is in keeping with previous observations where the brain was defined as a target tissue for *N. caninum* (Collantes-Fernández et al., 2006c; Almería *et al*., 2010Almería *et al*., 2016) but also in lung, skeletal muscle, heart and liver. Inflammatory infiltrate in foetal organs supports the hypothesis that at least partial foetal immunocompetence is already developed at this time, although no specific antibodies were found in foetal sera or in foetal fluids, as explained above.

In contrast to those results observed after infection with the isolate of high-virulence Nc-Spain7, the infection with the low-virulence isolate Nc-Spain1H did not induce foetal death, and only one G-NcSpain1H animal presented positive placental samples at 20 dpi, similar to a previous experimental study at early gestation (Rojo-Montejo *et al*., 2009b). In addition, there were no evident lesions at the placenta. Taken together, these results suggest a limited colonization of maternal placenta by Nc-Spain1H, which is consistent with the low proliferation rate of this isolate under *in vitro* conditions (Regidor-Cerrillo *et al*., 2011; Jiménez-Pelayo *et al*., 2017). In addition, four CO samples were positive, suggesting that as demonstrated *in vitro* in F3 cells, the foetal compartment of the placenta may be the target cell and the preferential niche for parasite multiplication, whereas caruncular cells seem to play a barrier role for the placenta, limiting the invasion and multiplication of the parasite (Jiménez-Pelayo *et al*., 2017). Moreover, higher activation of the innate immune responses, specifically TLR-2, on the maternal side as observed *in vitro* (Jiménez-Pelayo *et al*., 2019), may contribute to the elimination of the tachyzoites, diminishing the burden in the caruncle and limiting tissue damage. Despite the absence of parasite DNA, lesions or foetal antibodies in G-NcSpain1H foetuses at 20 dpi, the identification of parasite DNA on CO indicated the transmission of this isolate to the foetal compartment. In fact, the origin of this isolate (from a dairy herd with high intra-herd *N. caninum* seroprevalence) (Rojo-Montejo *et al*., 2009a) and a previous experimental infection at early gestation (Rojo-Montejo *et al*., 2009b) also corroborate that transmission of Nc-Spain1H to the foetus does occur. It is therefore tempting to hypothesize that if the experimental design of the study had allowed a longer gestation, the parasite might have been transmitted to the foetus.

Related to the pathogenesis of abortion, in the present work, higher parasite burdens and more severe lesions were detected in placentomes from one animal carrying NVF (3581) compared to VF, demonstrating that replication of the parasite at the maternal-foetal interface may be an important factor of foetal mortality (Buxton *et al*., 2002; Innes *et al*., 2007). On the other hand, resolution of placental lesions was demonstrated at 42 dpi (Maley *et al*., 2003), which indicated that progression of infection had been halted by the dam and the foetus and could be a reversible process in some cases. Moreover, our results showed that the %LES of the placenta was low, lesions showed a focal distribution and severity of the lesions did not seem sufficient to justify the foetal death by themselves because hypoxia signs were not found in NVF and placental functions did not seem to be compromised. There were no differences in the parasite burden in FB between VF and NVF, and only slightly higher parasite burden in FL was found in NVF. In addition, similar lesions were found in the foetal brain, liver, lung, heart and skeletal muscle of all Nc-Spain7 foetuses at 20 dpi. Brain lesions could be evaluated only in VF since NVF presented autolysis of the CNS. A key question that remains unsolved is the roles of the maternal and foetal immune responses in the outcome of the infection.

In summary, wider parasite dissemination with earlier transmission to the foetus and foetal death were found after infection with the high-virulent isolate Nc-Spain7 as soon as 10 and 20 dpi, respectively. All these findings seem to be related to a better capacity of this isolate to invade the placenta earlier and proliferate more efficiently. The pathogenesis of the abortion could not be determined with our findings, since placental and foetal burdens and lesions in VF and NVF would not explain by themselves the foetal death. Therefore, the roles of the maternal and foetal immune responses in the outcome of the infection should be investigated. However, this experiment was not designed to elucidate the cause of the abortion, and closer monitoring of the foetus and sequential sampling and culling are warranted in further research.

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Declarations

Ethics approval and consent to participate

All protocols involving animals were approved by the Animal Research Ethics Committee of the Principado de Asturias, Spain (reference number PROAE 25/2016), following the proceedings described in Spanish and EU legislations (Law 32/2007, R.D. 53/2013, and Council Directive 2010/63/EU). All animals were handled in strict accordance with good clinical practices and all efforts were made to minimize suffering.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JRC, PH, ECF, JB, KO and LMO conceived the study and participated in its design; LMO coordinated the study with the help of KO and obtained its funding. LJP and JB wrote the manuscript, with results interpretation and discussion inputs from PH, JRC, ECF, KO and LMO; JBM and ART performed animal reproductive and health management prior to infection. PH prepared the *inoculum*. LJP, MGS, PV and ART monitored the animals during the experimental period; LJP and MGS processed the samples during the experiment and after culling but histological processing and examination that were carried out by DGE and JB. LJP, MGS, PV, JB, DGE, ART and KO participated in the collection of samples during necropsy. LJP, MGS, JB and DGE carried out statistical analysis and interpreted the results. All authors read and approved the final manuscript.

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Additional File 1: Materials and Methods. Description of the health and reproductive handling of the cattle and the tissue DNA extraction and PCR determinations.

Health and reproductive handling of the cattle

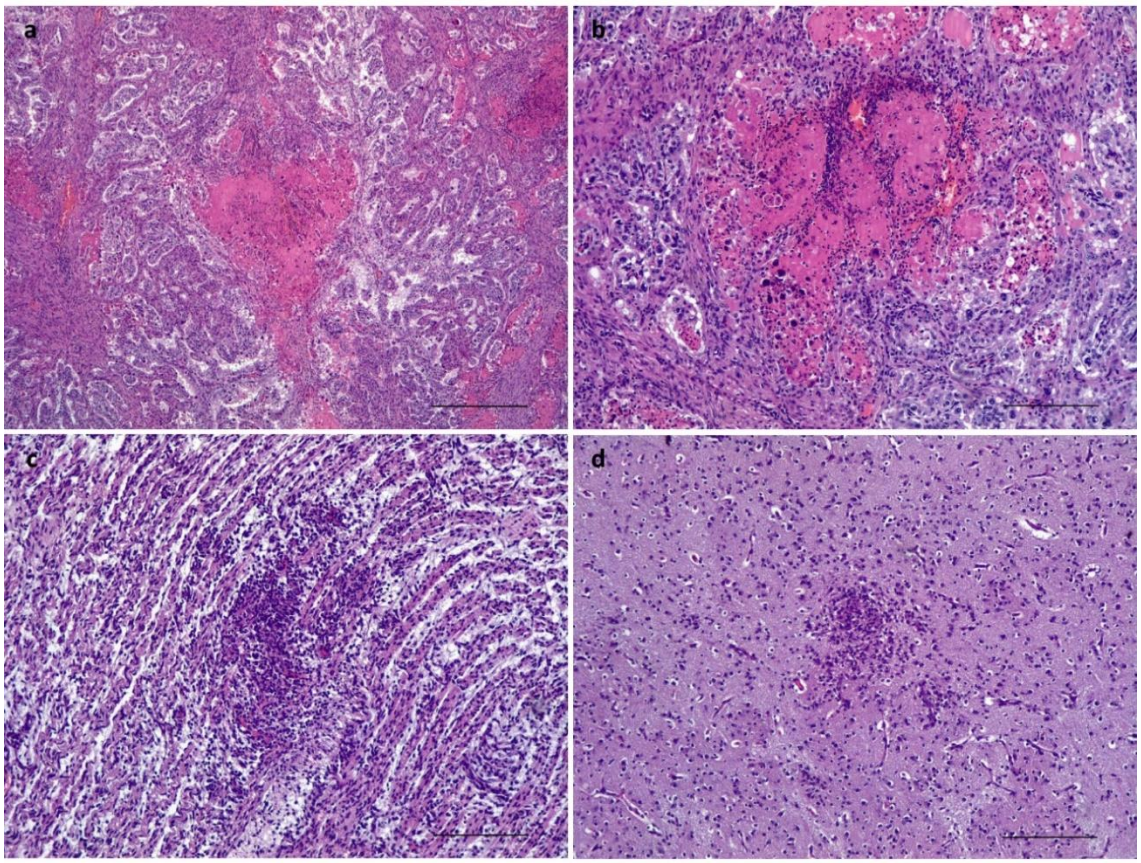
All animals were vaccinated against IBR virus and BVD virus with two doses of BOVILIS® BOVIPAST RSP (MSD Animal Health, Milton Keynes, UK), separated for 4 weeks, and treated with antiparasitic products (Endoex® and Albendex®, s.p. veterinaria, s.a., Riudoms, Spain; Animec Plus®, Cenavisa laboratorios, Tarragona, Spain) following the manufacturer's recommendations. Heifers were oestrus synchronised with the administration of: i) CIDR (Zoetis, New Jersey, USA) + 100 µg of synthetic gonadorelin analogue (Cystoreline®, CEVA, Barcelona, Spain) (day 0); ii) CIDR removing + 400 UI of equine serum gonadotropin (Folligon®, MSD Animal Health, Milton Keynes, UK) + 25 mg synthetic prostaglandin F2α analogue (Dinolytic®, Zoetis, New Jersey, USA) (day 5); iii) 25 mg synthetic prostaglandin F2α analogue (Dinolytic®, Zoetis, New Jersey, USA) (day 6); iv) 100 µg of synthetic gonadorelin analogue (Cystoreline®, CEVA, Barcelona, Spain) (day 7). Fifty-six hours after the first administration of PGF2α, two artificial insemination, 12 h apart, were carried out using semen from two Asturiana bulls seronegative to *N. caninum*. Pregnancy was confirmed by ultrasound scanning on day 35 after insemination, and twenty-four pregnant animals were selected for the experiment.

Tissue DNA extraction and PCR determinations

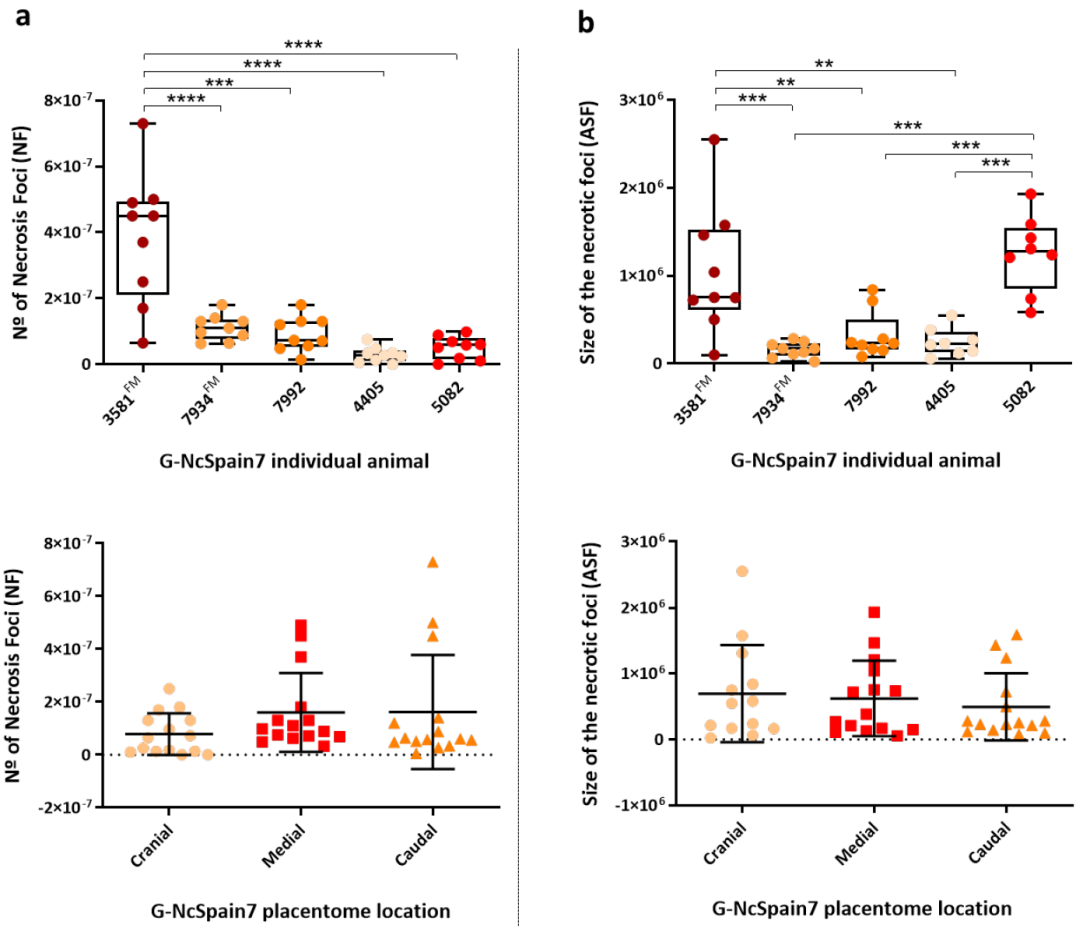
DNA extraction and PCR determinations were carried out as described elsewhere (Regidor-Cerrillo *et al*., 2014; Arran-Solís *et al*., 2015). Briefly, genomic DNA was extracted from 20-100 mg of maternal and foetal tissue samples using the Maxwell® 16 Mouse Tail DNA Purification Kit (Promega, Wisconsin, USA), following the manufacturer´s recommendations. The DNA concentration was determined for each sample using a Synergy™ H1 microplate reader (Biotek Instruments Inc, Winooski, VT, USA), and the samples were adjusted to 100 ng/µl in molecular grade water. Parasite DNA detection was carried out by nested PCR adapted to a single tube from the internal transcribed spacer (ITS1) region of *N. caninum* using TgNN1-TgNN2 as external primers and NP1-NP-2 as internal primers (Buxton *et al*., 1998; Hurtado *et al*., 2001; Regidor-Cerrillo *et al*., 2014). Each reaction was performed in a final volume of 25 µl with 5 µl of sample DNA. PCR was carried out in 9 samples of CA and 9 samples of CO as well as 3 samples of maternal pre-scapular and ileofemoral lymph node and 3 samples of FB and FL. DNA samples from G-Control were included in each round of DNA extraction and PCR as negative controls. Positive PCR controls with *N. caninum* genomic DNA equivalent to 10, 1 and 0.1 tachyzoites in 100 ng of bovine DNA were also included in each batch of amplifications. Fifteen µl aliquots of the PCR products were visualized under UV light in a 1.5% agarose/GelRed™ (Biotium INC) gel to detect the *N. caninum*-specific 247 bp amplification product.

Nested-PCR positive samples were adjusted to a concentration of DNA of 20 ng/µl, and quantification of *N. caninum* DNA was performed by real-time PCR using the equipment ABI 7500 FAST (Applied Biosystems, Foster City, CA, USA). The Nc-5 region was targeted as described elsewhere (Collantes-Fernández *et al*., 2005). A volume of 5 µL of diluted DNA from each sample was used for the qPCR assays. The number of *N. caninum* tachyzoites was determined by interpolating the Ct values (cycle threshold value) on a standard curve. The standard curve was designed for the quantification of 10^{-1} to 10^{5} tachyzoites according to Regidor-Cerrillo *et al*. (2011). To normalize the quantification of the parasite in each sample, a bovine β-actin standard curve was designed (from 64 ng of DNA per µl to 0.2 ng per µl). The results were expressed as the relation between parasite DNA and cell DNA amount ($R^2 \geq 0.99$; Slope values varied from -3.63 to -3.18).

Additional File 2. Histological findings in placental and foetal samples. (a) Placenta. G-NcSpain7 20 dpi. Three foci of necrotic placentitis with mild infiltration of inflammatory cells at the interdigitate area of the placentome HE. 4×. Bar 500 µm. **(b)** Placenta. G-NcSpain7 20 dpi. Focal necrosis with mild infiltration of inflammatory cells at the interdigitate area of the placentome. HE. 10×. Bar 200 µm. **(c)** Foetal heart. G-NcSpain7 20 dpi. Focal non-suppurative myocarditis. HE. 10×. Bar 200 µm. **(d)** Foetal brain. G-NcSpain7 20 dpi. Glia focus with a small area of necrosis at the centre of the lesions. HE. 10×. Bar 200 µm.



Additional File 3. Quantification of necrosis foci (NF), size (ASF) and affected area (%LES) of these foci. Graphs representing median number of cells, lower and upper quartiles (boxes) and minimum and maximum values (whiskers) of **(a)** number of NF in G-NcSpain7 culled at 20 dpi studied by individual animal or by placentome location, **(b)** ASF in G-NcSpain7 culled at 20 dpi studied by individual animal or by placentome location and **(c)** %LES in G-NcSpain7 culled at 20 dpi studied by individual animal, by placentome location or by placentome location in the placentomes of animals with higher ASF (3581 and 5082). *****, ****, ** and * symbols indicate $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$ significant differences.



Additional File 3. Continued.

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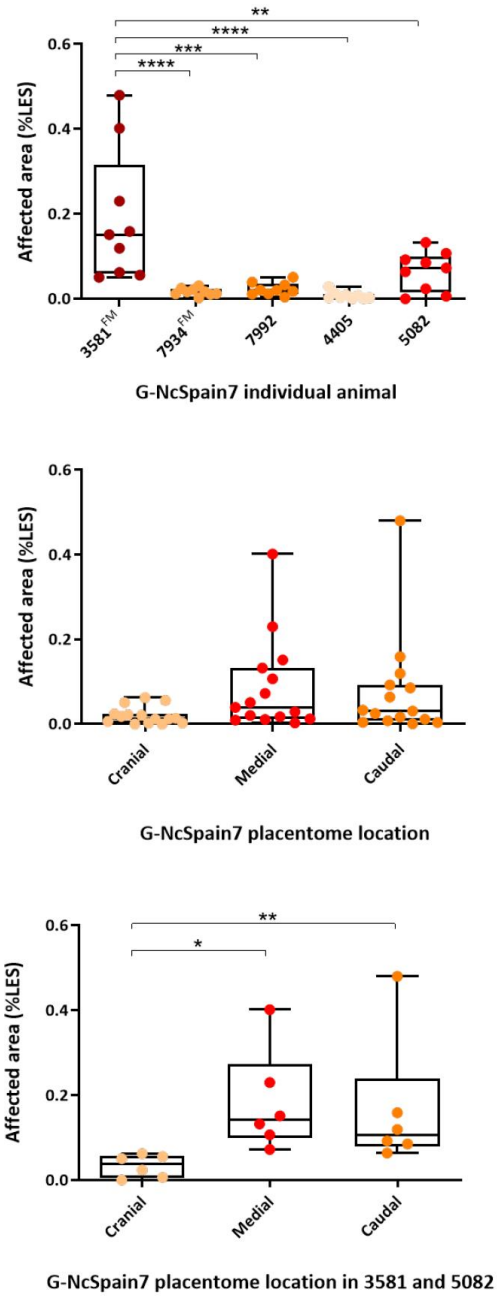


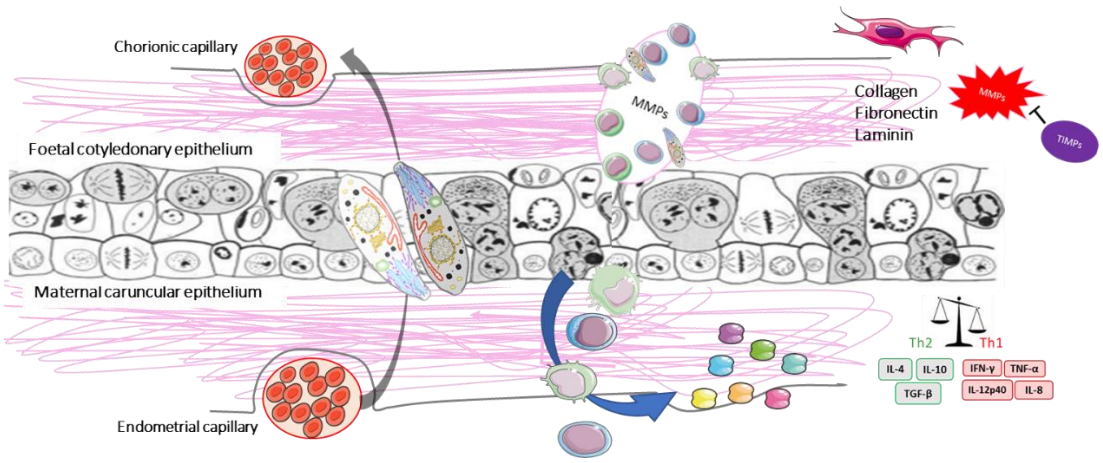
Sub-objective 2.2: Placental immune response and extracellular matrix organization during the early stages of *N. caninum* infection in pregnant heifers inoculated with high- and low-virulence isolates at mid-gestation.

Jiménez-Pelayo L[1], García-Sánchez M[1], Collantes-Fernández E[1], Regidor-Cerrillo J[2], Horcajo P[1], Gutiérrez-Expósito D[4], Espinosa J[4], Benavides J[4], Osoro K[4], Pfarrer C[5], Ortega-Mora LM[1]*.

[1]SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[2]SALUVET-innova, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitariia s/n, 28040 Madrid, Spain
[3]Instituto de Ganadería de Montaña (CSIC-Universidad de León), 24346, León, Spain
[4]Regional Service for Research and Agri-Food Development (SERIDA), 33300 Villaviciosa, Asturias, Spain
[5]Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany

*Corresponding author: Luis Miguel Ortega-Mora

Running title: **Differential immune responses and extracellular matrix organization are observed in the bovine placenta during early infection at mid-gestation with high- and low-virulence *Neospora caninum* isolates.**



Manuscript in preparation
This work has been accepted as a poster in the 5th International Meeting on Apicomplexa in Farm Animals (Apicowplexa) (2nd-4th October 2019, Berlin, Germany).

CHAPTER V

GENERAL DISCUSSION

DISCUSIÓN GENERAL

Neospora caninum is considered to be a major cause of abortion in cattle worldwide, and bovine neosporosis has become a major concern for the scientific community in the last years (Dubey *et al*., 2017, Ortega-Mora *et al*., in press). Important economic losses have been related to this infection, mainly associated with abortion, lengthened calving interval, reduced stock value and increased culling rate (Trees *et al.*, 1999; Reichel *et al.*, 2013)

Transplacental transmission is the main route of transmission in cattle, playing the bovine placenta a fundamental role in the pathogenesis of bovine neosporosis. Apart from its barrier function, the placenta can act as an immune regulatory organ. Trophoblasts and other cell types within the placenta may be involved in the physiological protection of the placenta, recognizing pathogens and leading to cytokine induction and regulation of co-estimulatory molecules (Montes *et al*., 1995; Steinborn *et al*., 1998a; Steinborn *et al*., 1998b). However, little is known about the interaction of the parasite with trophoblastic cells and other cell types in the placenta. Moreover, differences in the virulence of *N. caninum* isolates have been associated with variation in the clinical presentation of the disease. Nevertheless, the factors that enable some isolates to be more effectively transmitted and cause foetal death than others are still unclear. In this context, the initial events occurring after parasite infection are crucial. Recent transcriptomic and proteomic studies *in vitro* suggested a higher modulation of the host cell by the low-virulence isolate Nc-Spain1H and a different expression of genes involved in important metabolic, stress response and innate immune response processes (Regidor-Cerrillo *et al*., 2012; Horcajo *et al*., 2017; García-Sánchez *et al*., submitted). However, the mechanisms used by the parasite to modulate bovine host cells during the early phase of infection are not completely understood and the determining factor leading to foetal death is not already known. Four mechanisms have been postulated so far as possible cause of abortion: tachyzoites multiplication and the consequent damage in foetal organs; placental damage induced by the replication of the parasite; alteration of the immune balance at the placental level inducing placental damage; and prostaglandin secretion (Buxton *et al*., 2002; Innes *et al*., 2005; Dubey *et al*., 2006; Dubey *et al*., 2007; Innes, 2007). Here, the principal objective was to bring light to the role of the bovine placenta in pathogenesis of neosporosis and to identify potential pathways or biological networks at the maternal-foetal interphase that could explain why some isolates are more effectively transmitted and cause higher foetal death than others. In order to achieve this main objective, *in vitro* and *in vivo* investigations in placental target cells and in pregnant cattle have been carried out. Comparisons between high- and low-virulence isolates were carried out using two Spanish *N. caninum* isolates that previously demonstrated marked differences in virulence both *in vitro* and in mice and bovine models.

First studies of this Doctoral Thesis were carried out in established bovine caruncular and trophoblast cell lines. *In vitro* assays offer a reduction in the usage of animals for experimentation and represent powerful tools for the study of apicomplexan parasites (Muller & Hemphill, 2012). These models have enabled studies on parasite virulence, host factors involved in innate resistance, parasite stage conversion and drug effectiveness, among others (Muller & Hemphill, 2012). The use of established cultures has been widely accepted in order to provide an initial approach to certain mechanisms involved in complex processes since they allow the obtaining of reproducible results and the reduction of experimental animals as well as the optimization of the methods that will be used later during *in vivo* experiments. This is the first study where a species- and organ-specific *in vitro* model for each of the host cell layers in the maternal-foetal interface

of the bovine placenta has been implemented to study *N. caninum* infection in bovine placental cells. To date, several *in vitro* studies have been carried out using established cell lines, such as Marc-145, HeLa, BeWo or ovine trophoblast cells, to investigate the lytic cycle of different *N. caninum* isolates (Haldorson *et al*., 2005; Carvalho *et al*., 2010; Regidor-Cerrillo *et al*., 2011). There was only one limited, descriptive study concerning the interaction of the parasite with bovine trophoblast cells (Machado *et al*., 2007), and no data were available about the parasite interaction with bovine caruncular cells. Bovine trophoblast cells (F3) and bovine caruncular epithelial cells (BCEC-1) had been used previously for the study of drug transport across the maternal epithelium as part of the epitheliochorial placental barrier of the cow (Waterkotte *et al*., 2011), for the investigation of biological processes such as differentiation or invasion during placentation (Hambruch *et al*., 2010; Loch *et al*., 2018) or for the study of other abortifacient pathogen for cattle as *Coxiella burnetii* (Sobotta *et al*., 2017), showing a good reproducibility of the results. Results in this study have demonstrated that this model is very interesting for the study of *N. caninum* and a useful tool to evaluate critical factors involved in placental pathogenesis. In addition, BCEC-1 and F3 were isolated from fifth- and fourth-month pregnant heifers, respectively, and they have maintained at least part of their morphological and functional characteristics (Bridger *et al*., 2007b; Hambruch *et al*., 2010; Waterkotte *et al*., 2011). Thus, they are a good model for exploring *N. caninum* infection since transplacental transmission predominates in the second third of pregnancy, when the majority of abortions caused by *N. caninum* occur (Dubey *et al*., 2007).

To develop all the experiments, two *N. caninum* isolates were selected based on their virulence *in vitro* and *in vivo* and maintained in cell culture. The number of passages was controlled in order to minimize the influence of culture adaptation as was demonstrated that continuous culture passages of the tachyzoites may lead to an adaptation of the parasite, modifying its *in vivo* biological behaviour and virulence (Pérez-Zaballos *et al*., 2005; Bartley *et al.,* 2006).

First, the high virulent isolate Nc-Spain7 was obtained from an asymptomatic calf (Regidor-Cerrillo *et al*., 2008) that presented 100% of brain samples positives to *N. caninum* DNA and more than 50% of the brain samples with characteristic lesions. Later studies *in vitro* showed mild rates of invasion and high rates of proliferation in MARC-145 cells (Regidor-Cerrillo *et al*., 2010). Those results correlated with a high virulence observed in the pregnant murine model, where high maternal mortality and neonatal morbidity as well as high rates of neonatal mortality and vertical transmission were found (Dellarupe *et al*., 2014a). Recent studies in bovine macrophages showed high proliferation and survival of Nc-Spain7 (García-Sánchez *et al*., 2019). In the present Doctoral Thesis, *in vitro* assays in bovine placental cells showed higher invasion and infection rates and higher proliferation (higher tachyzoite yield and lower doubling time) of Nc-Spain7 in bovine trophoblast cells. GRA17 expression was higher in Nc-Spain7 than Nc-Spain1H, and in *T. gondii* this protein has been related to faster growth (Gold *et al*., 2015; Horcajo *et al*., 2018). In addition, gliding associated proteins GAP40, GAP50, GAPM3, as well as five inner membrane complex (IMC) proteins, had higher expression in Nc-Spain7, and have been associated with a role in parasite motility and host cell invasion (Frenal *et al*., 2010), and probably in proliferation (Horcajo *et al*., 2018). However, Nc-Spain7 invasion, concretely adhesion, and proliferation were dramatically lower in caruncular cells than in trophoblast cells. Trophoblast cells are the first layer of the foetus, and our results suggested a role as a niche for parasite multiplication. On the other hand,

the influence of the isolate on the cytokine profiles induced during the infection has been previously demonstrated (Rojo-Montejo *et al.*, 2009b; Caspe *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014). In a second work *in vitro* in bovine trophoblast and caruncular cells, the expression levels of molecules related to the immune responses and endothelial adhesion molecule genes were assessed after infection with both isolates at early and late stages post-infection. Several immune elements such as TLR-2, IL-6, IL-8, TNF-α and TGF-β1, showed to be expressed for trophoblast and caruncular cell cultures without any extra stimulus but the parasite, proving that placental cells are early responders to the infection and are implicated in defence against the parasite and participate in local immune responses. Those results were confirmed later through *in vivo* investigations, and recent *in vitro* experiments with human syncytiotrophoblast and cytotrophoblast infected by *T. gondii* reported comparable results (Ander *et al.*, 2017). In addition, slight differences between isolates could be also detected, and observations made *in vitro* suggested an evasion strategy of Nc-Spain7 to avoid immune responses, with diminished expression of TLR-2 and TNF-α compared to Nc-Spain1H.

The low-virulence isolate Nc-Spain1H was also isolated from a clinically healthy calf with precolostral *N. caninum* antibodies from a dairy herd with high *N. caninum* intraherd seroprevalence (Rojo-Montejo *et al.*, 2009a). *In vitro* experiments suggested a low invasion and proliferation rate of this isolate (Rojo-Montejo *et al.*, 2009a; Regidor-Cerrillo *et al.*, 2010), later confirmed in the pregnant murine model. Presence of Nc-Spain1H in maternal tissues and maternal morbidity were low. In addition, only 5% of vertical transmission and 0.5% of neonatal mortality were observed (Rojo-Montejo *et al.*, 2019; Pereira García-Melo *et al.*, 2010). In bovine macrophages Nc-Spain1H also presented lower proliferation and survival (García-Sánchez *et al.*, 2019). Here, the low virulence isolate showed lower invasion and proliferation in trophoblast cells than the Nc-Spain7 isolate. In fact, previous proteome studies revealed a patron similar to a pre-bradyzoite stage for Nc-Spain1H, associated with low proliferative isolates in *T. gondii* (Horcajo *et al.*, 2018). In caruncular cells, as observed with the high-virulence isolate, Nc-Spain1H showed a remarkable reduced adhesion and proliferation, and differences between both isolates were minor in this cell line. Those results suggested a particularly remarkable feature of the bovine placenta with the presence of both permissive and resistant cells in very close proximity, indicating that cell autonomous features determine the fate of *N. caninum* upon interaction with trophoblast and caruncular cells. *In vitro* infection of trophoblast and caruncular cells showed that trophoblasts are the niche for parasite proliferation whereas caruncular cells seem to restrict the infection. Moreover, our *in vitro* investigations suggested that caruncular cells limited *N. caninum* infection at two critical stages of the parasite lytic cycle—at the point of parasite adhesion to the host cell and during intracellular growth. Parallel results in human second-trimester syncytiotrophoblasts and cytotrophoblasts infected by *T. gondii* showed reduced adhesion and replication of *T. gondii* in syncytiotrophoblasts, as the primary placental barrier formed by cells in direct contact with maternal blood. Nevertheless, high proliferation of the parasite was observed in cytotrophoblasts, as the subjacent migratory layer, supporting our result (Ander *et al.*, 2017). Finally, in the second work, Nc-Spain1H showed higher stimulation of TLR-2 and TNF-α than Nc-Spain7, suggesting a better control of its multiplication by the immune system which may be an advantage to be transmitted to the progeny without causing foetal death and abortion.

Despite of the importance and great utility of *in vitro* models, they do not consider information on environment influence. Hence, *in vivo* models are essential for the study of several aspects that otherwise could not be assessed by means of *in vitro* culture, being helpful for studies related to host-parasite relationship, immune response or pathogenesis (Reichel & Ellis, 2009). Results obtained *in vitro* were consolidated and complemented by studying the early consequences of infection (10 and 20 dpi) in a pregnant bovine model inoculated with high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *N. caninum* at mid-gestation. Although abortion caused by *N. caninum* is more frequent during the second trimester of pregnancy in naturally infected cattle (Dubey *et al*., 2007) only few pregnant bovine models of neosporosis at this gestation time have been reported (Maley *et al*., 2003; Bartley *et al*., 2004; Almería *et al*., 2010; Almería *et al*., 2016a). On the other hand, the consequences of either primoinfection or recrudescence in a pregnant cow can be abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf. Many factors are implicated in the outcome of the infection, related to the host (gestation period, maternal and foetal immune responses, origin of the infection and breed) and the parasite (isolates and intraspecific variability, parasite stage, inoculation route and dose of infection). Parasite biological variability is one of the main factors influencing the outcome of the infection.

Specifically, the high-virulence isolate Nc-Spain7 showed a percentage of abortion and vertical transmission of 100% in a bovine model at early gestation (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014), whereas the infection in experimentally infected cattle with the low-virulence isolate Nc-Spain1H did not result in foetal death (Rojo-Montejo *et al*., 2009b). Later studies carried out at mid-gestation showed that intravenous inoculation of 10^7 tachyzoites of Nc-Spain7 produced 50% foetal death until 42 dpi (Almería *et al*., 2016a) and 66.6% foetal death when gestation lasted until term. A recent work focused on the standardization of a pregnant bovine model of neosporosis during the second third of gestation (Vázquez *et al*., submitted), concluding that intravenous inoculation of 10^7 tachyzoites from the high virulent Nc-Spain7 isolate could be an adequate route and dose combination with more than 50% of abortions and efficient parasite transplacental transmission (100%) (Vázquez *et al*., submitted). However, early events occurred after the arriving of the parasite to the placenta and comparison between different virulence isolates inoculated at mid-gestation had not been investigated until now. Serial culling at 10 and 20 dpi of pregnant cattle infected by high- and low-virulence isolates was carried out in order to investigate the early infection dynamics as well as early immune responses and ECM modulation induced locally at the placenta *in vivo*. Results from the present Doctoral Thesis confirmed differences in virulence between isolates during infection in heifers at mid-gestation and suggested differences in the proliferation and modulation of the placental immune responses and the ECM by the high virulent isolate Nc-Spain7 and the low virulent isolate Nc-Spain1H.

The first clinical sign detected in Nc-Spain7 animals was an increment in body temperature at 1 dpi in half of the animals, in agreement to previous reports (Buxton *et al*., 1998; Williams *et al*., 2000; Macaldowie *et al*., 2004; Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014; Almería *et al* 2016a; Vázquez *et al*., submitted). In addition, only Nc-Spain7 infection induced a second peak of fever at 3 dpi, indicating earlier and higher replication of this isolate, leading to a second antigenic exposition of Nc-Spain7 tachyzoites. Moreover, fever was detected in all animals infected with the high virulent isolate along the experimental time. Higher febrile responses induced by the high-virulence isolate may be explained by a more efficient multiplication of Nc-Spain7 in host

tissues as indicated previously (Regidor-Cerrillo *et al*., 2010; Regidor-Cerrillo *et al*., 2011; García-Sánchez *et al*., 2019), and confirmed in placental cells *in vitro* as a part of the investigations of the present Doctoral Thesis.

Peripheral immune responses, cellular and humoral, were assessed in dams´ sera along the experimental infection, detecting a peak of IFN-γ in both infected groups at 2 dpi. Therefore, infection with *N. caninum* tachyzoites activated the cellular immune response, crucial for host defence to intracellular pathogens (Innes *et al*., 2002; Innes *et al*., 2005; Williams *et al*., 2007). However, despite Nc-Spain7-infected animals showed the highest IFN-γ levels in maternal sera, they did not correlate with protection against infection since Nc-Spain7 was detected earlier in the placenta and showed a more effective invasion and proliferation than the Nc-Spain1H isolate. On the other hand, specific antibodies against *N. caninum* were detected in all Nc-Spain7 heifers between 9 and 13 dpi, slightly earlier than in previous works (Maley *et al*., 2004; Almería *et al*., 2010; Almería *et al*., 2016a), and earlier than in Nc-Spain1H, probably associated again with the higher proliferation of this isolate.

Nc-Spain7 arrived at the placenta at **early stages of infection (10 dpi),** with 5 placental samples positives to *Neospora* DNA by PCR, and one of them showed focal necrosis, demonstrating the colonization of the placenta by this isolate. Similar to our observations, focal necrosis was described in placentomes at 2 wpi with the Nc-Spain7 isolate (Almería *et al*., 2016a). Early detection of Nc-Spain7 at 10 dpi may be associated with its higher invasion and proliferation ability in placental cells, as demonstrated *in vitro*. However, immunohistochemical labelling of *Neospora* antigen in placentomes revealed absence of detection of parasite antigen, probably due to the limited sensibility of IHC analyses and reduced burdens reached by the parasite at this time.

N. caninum was detected in the liver of two Nc-Spain7-infected foetuses, indicating that the high-virulence isolate is already transmitted to the foetus at 10 dpi, although lesions in foetal tissues were not found at this time. Previous studies have suggested the crossing and presence of tachyzoites in foetal tissues as early as 10 dpi (Barr *et al*., 1994; Maley *et al*., 2003). In addition, our findings may suggest that liver is the first target organ in the foetus when arriving from the placenta, which most probably represents the gateway for the parasite to invade the foetus through the umbilical vein, replicating in the parenchyma and after, spreading through the foetal body as was previously observed in sheep (Arranz-Solís *et al*., 2016). However, antibodies against *N. caninum* were not found in serum or corporal fluids from any foetus, probably because at least 6 weeks after infection are needed to detect antibodies in infected foetuses (Caspe *et al*., 2012; Almería *et al*., 2016a).

Immune response profiles in the bovine placenta were also investigated during this experimental infection at 10 dpi. The highly virulent isolate Nc-Spain7 showed apparently the capacity to evade the immune responses in the placenta. Only IFN-γ, TNF-α and IL-4 were moderately upregulated whereas expression levels of PRRs, chemokines and endothelial adhesion molecules, the rest of cytokines and ECM components were not modified. In addition, *in vitro* results from this Doctoral Thesis demonstrated a down-regulation of TLR-2 in bovine trophoblast and caruncular cells infected with Nc-Spain7 at early time-points post-infection respect to Nc-Spain1H-infected cells

as was demonstrated *in vivo*. Host cell modulation leading to evasion of local immune responses probably contributed to the more effective proliferation of Nc-Spain7 observed in placental tissues both *in vitro* and *in vivo*. In a similar way, Nc-Spain7 showed a higher ability to evade the host macrophage responses which, in conjunction with its higher replication and transmigration capacity, was associated with the higher virulence *in vivo* (García-Sánchez *et al*., 2019). Despite the lack of activation of classical PRRs involved in *N. caninum* detection at the placental level, increment in IFN-γ, TNF-α and IL-4 as well as T CD4+, CD8+ and B lymphocytes at 10 dpi suggest that other pathways of the immune system should be activated by the high virulent isolate. Other effector molecules of the innate immune response, as NLR proteins (NLRP12, NAIP, NLRC4), have been recently involved in the activation of the inflammatory responses in bovine macrophages after Nc-Spain7 and Nc-Spain1H infection *in vitro* (García-Sánchez *et al*., submitted). A higher concentration of Nc-Spain7 antigens or host cell components released during cell damage may be necessary to activate classical PRRs in the placenta during Nc-Spain7 infection. Further studies are necessary to identify new activation routes of the innate immune responses. Contrary to *in vivo* observations, IL-4 and IFN-γ were not expressed by bovine placental cells *in vitro*, showing that probably only T lymphocytes and other immune cells are implicated in their secretion after infection.

Interestingly, CD8+ cells prevailed over CD4+ cells at 10 dpi both in infected and control groups as was previously observed in sheep (Arranz-Solís *et al*., 2016). Previous studies in cattle and buffaloes found the opposite (Maley *et al.*, 2006; Rosbottom *et al.*, 2011; Cantón *et al.*, 2013; Cantón *et al*., 2014c), and CD4+ cells have been shown to be more relevant in the protection against neosporosis (Tanaka *et al.*, 2000a). However, our results may be related with an early response to *N. caninum* infection since it was suggested that CD8+ cells could predominate over CD4+ as early responders to *N. caninum* (Correia *et al.*, 2013). The increases in the immune cell populations were mainly associated to the maternal compartment of the placentome, a common finding given that *N. caninum* reaches the maternal caruncular septa *via* the circulation before infecting the foetal trophoblast villi cells and spreading to the foetus (Dubey *et al*., 2006). Therefore, it seems reasonable to assume that parasite is firstly recognized in the maternal part of the placenta (Marin *et al*., 2017), stimulating quickly the maternal immune response, which attracts inflammatory cells to the infection focus trying to control parasite dissemination to the foetus.

Culling at 20 dpi revealed foetal death in two Nc-Spain7-infected heifers in agreement with previous experimental infections (Almería *et al*., 2016a; Vázquez *et al*., submitted). Essentially 100% of caruncle and cotyledon samples were positivity to *Neospora* DNA, evidencing one more time the "tropism" of *N. caninum* for the bovine placental tissues, which seems to be one of the most appropriate niches for its multiplication. However, parasite was only observed in foetal villi of Nc-Spain7-infected animals at 20 dpi by IHC, as postulated *in vitro* that trophoblast cells are the real target of *N. caninum*.

Placental necrosis was observed in all NcSpain7-infected animals at 20 dpi associated with high parasite burdens. In addition, the extravasation of proteinaceous material and cellular debris found in the haemophagus area of the placentomes was larger at 20 dpi than at 10 dpi in Nc-Spain7-infected heifers and larger than in NcSpain1H-infected animals, suggesting a correlation

between the presence and severity of this histological change and infection by *N. caninum*, since larger areas of extravasation were observed in animals with higher parasite burden and infected with more virulent isolates. In fact, multiplication of the parasite in the endothelium might damage the placental vasculature, increasing vascular permeability and, thus, facilitating serum infiltration. Increased expression of SERP-1, as a component of the complement and coagulation cascades, was observed in infected placentomes from the *in vivo* model implemented in the present Doctoral Thesis, confirming complement and coagulation cascades modulation by the infection. In addition, those expression levels were higher at 20 dpi and higher in placentomes from non-viable foetuses than in viable foetuses, which may suggest an implication in foetal death. However, the role of such modulation in the pathogenesis of bovine neosporosis should be investigated more in depth.

Comparing parasite burden and lesion development in placentomes from animals carrying viable and non-viable foetuses, higher parasite burden in caruncles and cotyledons from heifers carrying non-viable foetuses suggested that a higher replication of the parasite took place in animals with foetal death. However, similar lesions in caruncles and cotyledons were found in all Nc-Spain7-infected heifers at 20 dpi. Moreover, the total affected area of the placenta was limited, lesions showed a focal distribution, and hypoxia or other signs of placental dysfunction were not found in non-viable foetuses. Therefore, our theory is that in addition to the placental damage, other factors are probably implicated in the foetal death.

In relation to the foetal tissues, almost 100% of brain positive samples were found at 20 dpi, as well as inflammatory infiltrate and lesions compatible with *N. caninum* in foetal CNS, which is in keeping with previous observations that described the establishment and persistence of the parasite in the CNS, where *N. caninum* transforms into the tachyzoite-bradyzoite stage inside tissue cysts (Buxton *et al.,* 2002). Viable and non-viable foetuses presented similar parasite burdens in the CNS with increasing charges along the experiment. Parasite burden in foetal liver tended to a decrease as have been previously postulated that foetal liver would act as the first target organ in the foetus to spread to immunopriviledged tissues. However, foetuses at mid-gestation are able to control and clear parasite infection at the liver (Buxton *et al.*, 1998; Arranz-Solís *et al*., 2016), being an evidence of specific foetal immunocompetence at this gestation period, although no specific antibodies were found in foetal sera or in foetal fluids. Certain foetal immunocompetence may be a possible cause of the fewer foetal death observed in our work compared to the infection earlier in gestation (Regidor-Cerrillo *et al*., 2014). Brain lesions could not be compared between viable and non-viable foetuses since non-viable foetuses presented autolysis of the CNS. Slightly more acute lesions were found in liver and lung from non-viable foetuses, that presented mild multifocal necrotic foci in addition to the inflammation also observed in viable foetuses. Mild mononuclear myositis and myocarditis were also found in all these five foetuses. As observed in the placenta, differences in burden and lesions between viable and non-viable foetuses did not seem enough by themselves to explain the foetal death observed in two foetuses.

An immune-mediated pathogenesis has been suggested as a possible cause of abortion (Quinn *et al*., 2002b). It has been shown that the multiplication of the parasite in the placenta alters the immunological balance at the maternal-foetal interface and this alteration depends on the isolate

implicated in the infection (Rojo-Montejo *et al.*, 2009b; Caspe *et al.*, 2012; Regidor-Cerrillo *et al.*, 2014). In the view of the evasion of the immune responses at 10 dpi by Nc-Spain7, we hypothesize that the intrinsic ability of rapid multiplication of this isolate led to a profound tissue damage with liberation of host cell components, which could be responsible for PRRs activation at 20 dpi. In consequence, the expression of all the pro-inflammatory cytokines and chemokines, specially of IFN-γ, TNF-α and IL-12p40, which correlated with an extensive inflammatory infiltrate of T CD4 +, T CD8+cells and macrophages, was dramatically enhanced. Contrary to observed in control and Nc-Spain1H-infected placentas, CD8+ still prevailed over CD4+ in Nc-Spain7-infected samples at 20 dpi. As indicated above, CD4/CD8 ratio is usually > 1 in cattle infected by *N. caninum*, in contrast to observations made in sheep (Arranz-Solís *et al.*, 2016). Although the causes of this predominance remain unknown, it has been postulated that a ratio < 1 in cattle is associated to an early stage of the infection. However, it cannot be ruled out that this difference may be related to the isolate employed in the view of the results presented in this Doctoral Thesis. Remarkably, placental samples infected with the high virulent isolate showed a predominant Th1 response at 20 dpi, with 10-fold less IL-4 than IFN-γ. IFN-γ is required to limit parasite proliferation (Innes *et al.*, 1995; Baszler *et al.*, 1999; Innes *et al.*, 2002), although a critical threshold of the IFN-γ response is also required to limit adverse effects on pregnancy (Almería *et al.*, 2017). These results may indicate Th1/Th2 deviation towards a Th1 predominance as one possible cause of the foetal death. In addition to Th1/Th2 balance, the increases of other local pro-inflammatory cytokines (Il-12p40, IL-8, TNF-α), iNOS and immune cells were associated with more-severe histopathological changes *in vivo* (Rosbottom *et al.*, 2008; Regidor-Cerrillo *et al.*, 2014; Arranz-Solís *et al.*, 2016), and exacerbated immunological reactions, particularly in the placenta, have been previously postulated as one of the causes of abortion (Raghupathy, 1997; Quinn *et al.*, 2002b; Almería *et al.*, 2010). Moreover, the number of T and B lymphocytes and phagocytic cells was higher in placentomes from animals carrying non-viable foetuses and molecules such IL-8, iNOS and TNF-α were up-regulated in those animals, supporting the hypothesis that exacerbate immune responses in Nc-Spain7-infected placentas may contribute to the foetal death. On the other hand, Nc-Spain7 multiplication and the consequent cell lysis led to a profound alteration of the ECM organization that may facilitate its direct transmission to the foetus but may also be implicated in the mechanism of foetal death. Loss of fibronectin, vimentin and collagen in focal necrotic areas together with down-regulation of MMP-2, MMP-14 and TIMP-2 in Nc-Spain7-infected placentas from animals carrying non-viable foetuses may indicate that the high virulent isolate can inhibit tissue repair, given that IL-17A and TGF-β1 have synergistic properties increasing IL-6 production during fibrosis processes (Dufour, 2018), and stimulating the release of procollagen and fibronectin (Choy & Rose-John, 2017). In addition, absence of IL-6 and IL-17A modulation by Nc-Spain7 may be understood as a part of the evasion strategy of this isolate, given that both cytokines have been associated with potent pro-inflammatory properties against *N. caninum* infection (Pinheiro *et al.*, 2010; Almería *et al.*, 2011; Flynn & Marshall, 2011; Peckham *et al.*, 2014). The lack of stimulation by Nc-Spain7 was also observed in placental cells *in vitro* and in bovine macrophages (García-Sánchez *et al.*, submitted). Interestingly, TGF-β1 was diminished in placentomes from animals carrying non-viable foetuses compared to viable foetuses, as was observed *in vitro* both in trophoblast and caruncular cells. TGF-β1 dysregulation might also contribute to the foetal death observed in Nc-Spain7 heifers since it is crucial in neutralizing the Th1 inflammatory responses (Entrican, 2002). Chemokines and endothelial adhesion molecules genes were also upregulated in Nc-Spain7-infected placentomes, associated with an extensive

inflammatory infiltrate of macrophages and T cells and severe histopathological changes. Although CCLs expression have been linked to protection against *N. caninum* infection (Mineo *et al*., 2010; Kameyama *et al*., 2012; Abe *et al*., 2015), exaggerated levels of these molecules were related to an increased incidence of abortion *via* increasing iNOS (Brandonisio *et al*., 2002; Menezes-Souza *et al*., 2012) or the inflammatory infiltrated in damaged areas (Pfaff *et al*., 2005; Wang *et al*., 2007; Ferro *et al*., 2008).

As introduced above, low virulence traits were observed in the Nc-Spain1H isolate *in vitro* and in mice model, and infection of pregnant heifers early in gestation did not lead to foetal death, although transmission was observed (Rojo-Montejo *et al*., 2009a; Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2010). In the experimental infection in pregnant heifers carried out during this Doctoral Thesis, the low-virulence isolate showed dissimilar early infection dynamics and immune response and ECM modulation that those described for the high-virulence isolate.

The low virulent isolate Nc-Spain1H induced fever quickly after inoculation (1 dpi). However, only half of the animals presented fever along the experimental period and a second peak of fever was not detected in these animals compared to Nc-Spain7-infected heifers. In addition, lower systemic IFN-γ levels and delayed seroconversion respect to the high virulent isolate were observed *in vivo*. It is unknown if the immune response developed in Nc-Spain 1H-infected animals was able to reduce parasite burden, limiting the tissue damage or if the low capacity of the isolate to multiply in host tissues may be associated with the reduction or absence of repeated antigenic stimulus (Buxton *et al*.,1991; Rojo-Montejo *et al*., 2009b; Regidor-Cerrillo *et al*., 2014).

Presence of Nc-Spain1H was not found in any placental or foetal tissue, and specific humoral responses were not found either in Nc-Spain1H-infected foetuses at 10 dpi. Lesions were not found either in placentas or in foetuses from animals infected with the NcSpain1H isolate, apart from serum extravasation in placentomes. However, intravenous inoculation is supposed to disseminate the parasite quickly through the organism. We hypothesize that the low rate of multiplication of Nc-Spain 1H tachyzoites, observed in placental tissues *in vitro*, especially in caruncular cells, where the growth of this isolate is clearly slow, might imply a low level of parasitaemia and a low risk of placental infection.

Interestingly, despite the absence of parasite detection, Nc-Spain1H induced a solid Th1 response at early stages of infection (10 dpi), that remained steady at later stages (20 dpi). Clues of this rapid activation of the pro-inflammatory responses by the low-virulence isolate were already found *in vitro*, with higher levels of TLR-2, TNF-α and IL-8 in trophoblast and caruncular cells infected with Nc-Spani1H than with Nc-Spain7 at early times post-infection. In addition, this observation agrees with previous proteome and transcriptome studies where the low-virulence isolate induced higher changes in host cells (Horcajo *et al*., 2017; Horcajo *et al*., 2018; García-Sánchez *et al*., submitted), and a higher stimulation of the immune responses from bovine macrophages *in vitro* compared to the Nc-Spain7 isolate (García-Sánchez *et al*., 2019). Th1 responses in Nc-Spain1H-infected placentas would be counterbalanced by a higher expression of anti-inflammatory and regulatory cytokines, minimizing pathology. These results could explain the more effective control of the parasite, thereby contributing to its lower proliferation, and suggest a better adaptation of the low virulence isolate to replicate in the placenta without compromising gestation, maintaining a delicate balance between suppression and induction of the host immune response to ensure foetal survival and vertical transmission. How placental

tissues can recognize and mount an appropriate immune response against this isolate without parasite detection is a fascinating question that remains unknown. Existence of soluble factors of Nc-Spain1H might contribute to earlier Nc-Spain1H recognition, as has been previously suggested for the interaction between other pathogens and some PRRs (Martínez-Colón *et al*., 2019). For example, higher expression of TLR-9 is observed in caruncular samples infected with Nc-Spain1H than Nc-Spain7 at 20 dpi, when Nc-Spain7 or the products derived from tissue damage have already been recognized by the rest of the PRRs, supporting the hypothesis that there are differences in the antigen exposition between high and low virulent isolates as well as different activation routes of the innate responses. TLR-9 expression by antigen presenting cells is essential for initiating the innate responses and developing an effective Th1-type immune response after *T. gondii* infection (Minns *et al*., 2006), and a recent transcriptomic work showed its activation in bovine macrophages after *N. caninum* infection (García-Sánchez *et al*., submitted). Higher expression of highly immunogenic SRS membrane proteins was observed in Nc-Spain1H than in Nc-Spain7 (Horcajo *et al*., 2018), supporting our theory of differential antigen exposition.

Contrary to Nc-Spain7, Nc-Spain1H induced a balanced or even predominant Th2 response in terms of IL-4 and IFN-γ. This observation may explain the absence of foetal death observed here and in previous experimental infections with this low virulent isolate (Rojo-Montejo *et al*., 2009b), together with the lower proliferation and the absence of lesions in the Nc-Spain1H-infected placentas and foetuses. Therefore, differences in the Th- responses between isolates may be a cause of the different outcome observed. Apart from IFN-γ, other pro-inflammatory cytokines, as IL-1β, TNF-α, IL-12p40, IL-8 and IL-6, showed an important implication in host defence during *N. caninum* infection in the placenta (Entrincan, 2002; Rosbottom *et al*., 2008; Almería *et al*., 2011). Interestingly, IL-6 and IL-17A expression levels were only increased in Nc-Spain1H-infected placentas at 10 dpi, decreasing at 20 dpi. IL-6 and IL-17A might be relatively important in controlling *N. caninum* in the first moments post-infection, and the relation between IL-6, IL-17A and TGF-β1 may contribute to lesions resolution, which contributes to maintain the homeostasis of the bovine placenta. Together with IL-4 and TGF-β1, IL-10 increase in caruncle counter-regulates pro-inflammatory cytokines at the maternal-foetal interface to avoid rejection (Innes, 2007), although it allows parasite proliferation and vertical transmission. Curiously, a lower expression of IL-10 was detected in cotyledonary samples infected by both parasites. Although specific research is needed, IL-10 downregulation in foetal cotyledons could implicate an intrinsic control mechanism of foetal trophoblast against external aggressions. Rapid upregulation of chemokines and cytokines may be associated with a slight increment of macrophages in Nc-Spain1H placentomes at 10 dpi. Macrophages, the first defensive mechanism against bovine neosporosis, may contribute to parasite migration (Lambert *et al.*, 2006; Lachenmaier *et al.*, 2011; Furtado *et al.*, 2012; García-Sánchez *et al*., 2019), acting as "Trojan horse". In fact, recent finding demonstrated an enhanced motility of bovine macrophages infected with *N. caninum* (García-Sánchez *et al*., 2019). ICAM-1, also upregulated by Nc-Spain1H, is involved in paracellular transmigration of *T. gondii* (Barragan *et al*., 2005), facilitating parasite dissemination and vertical transmission. Therefore, paracellular migration and "Trojan horse" strategy may be important for parasite dissemination *in vivo*, in particular into immune-privileged tissues as the placenta and in low-virulence isolates (Barragan *et al*., 2005). Interestingly, our results showed upregulation of ECM modulators by Nc-Spain1H, as observed in trophoblast cells *in vitro* (Horcajo *et al*., 2017), and lack of ECM components alteration. ECM remodelling may also help to the arriving of

leukocytes in infected areas and host MMPs are involved in infected macrophage dissemination (Seipel *et al*., 2010). Moreover, MMPs and TIMPs modulation by the infection might be involved in the crossing through the placental barrier (Wang & Lai, 2013), and it has been postulated that ameboid migration of infected macrophages diminished collagen destruction (Olafsson *et al*., 2018; García-Sánchez *et al*., 2019). Therefore, we hypothesize that ECM modification, together with the "Trojan horse" mechanism, may be used by the low virulent isolate in order to facilitate its passage throughout the placenta and be transmitted to the foetus.

At 20 dpi, only one Nc-Spain1H-infected animal presented positive placental samples (5/9). From these 5 positive samples, only one caruncular sample was *N. caninum* DNA positive whereas four samples from cotyledon were positive in the animal infected with Nc-Spain1H that presented positivity in the placenta, confirming transmission of this isolate and, on the other hand, supporting findings made *in vitro* which suggested the presence of both permissive and resistant cells in very close proximity in the placenta. Low presence of Nc-Spain1H in placental samples was similar to a previous experimental study at early gestation (Rojo-Montejo *et al*., 2009b), and contrary to observed during infection with the high-virulence isolate Nc-Spain7 (Regidor-Cerrillo *et al*., 2014). In addition, there were no evident lesions at the placenta in Nc-Spain1H-infected animals at 20 dpi. Taken together, these results suggest a limited colonization of maternal placenta by Nc-Spain1H, which is consistent with the low proliferation rate of this isolate under *in vitro* conditions. Moreover, earlier activation of the innate immune responses observed *in vivo* and higher activation of TLR-2 observed *in vitro*, may also contribute to the development of an earlier and more efficient immune response for elimination of the tachyzoites, diminishing the burden in the caruncle and limiting tissue damage as postulated above.

On the other hand, transmission of Nc-Spain1H to the foetal compartment occurs, although no parasite DNA, lesions or foetal antibodies were found in Nc-Spain1H-infected foetuses. However, the origin of this isolate and a previous experimental infection at early gestation corroborated the existence of transmission (Rojo-Montejo *et al*., 2009a; Rojo-Montejo *et al*., 2009b). As described above, exacerbated immune responses were not observed in Nc-Spain1H placentas. In fact, Th1/Th2 responses were always balanced or even deviated towards Th2 predominance in Nc-Spain1H-infected placentomes, and anti-inflammatory cytokines such as TGF-β1, crucial to maintain gestation, was always upregulated. In addition, expression levels of ICAM-1, chemokines and ECM modulators were maintained at 20 dpi and might implicate that Nc-Spain1H crosses the placental barrier by hijacking immune cells in a mechanism similar to a "Trojan horse". It is therefore tempting to hypothesize that if the experimental design of the study had allowed a longer gestation, probably Nc-Spain1H had not induced foetal death and abortion contrary to Nc-Spain7 since its strategy to be transmitted seems to be based on higher modulation of the extracellular matrix and probably a higher transmission *via* immune cells, limiting tissue damage and abortion.

In summary, differences between isolates are related to higher invasion and proliferation abilities of the Nc-Spain7 isolate and to the existence of an evasion mechanism consisting of a delayed activation of the immune responses of the high-virulence isolate in placental tissues compared to the low-virulence isolate. We propose that Nc-Spain7 arrives to the foetus using its efficient replication ability and an evasion of the placental immune response strategy in the early stage of

the infection. However, high multiplication leads to placental damage and exacerbated immune responses that may be the causes of the abortion. Higher proliferation and lower activation of the immune responses may be associated with higher expression of certain proteins as ROP17 or glideosome proteins and the lack or the minor expression of certain highly immunogenic SRS proteins in the high-virulence isolate, respectively. Virulence may be beneficial if the goal of the parasite is to kill the host and to increase the chances of ingestion and infection of the second host, as may be happening with Nc-Spain7, that would exploit its intrinsic invasion and replication capabilities as well as an early evasion of the placental immune responses in order to arrive to the foetus, leading to alteration of the local Th1/Th2 balance, placental damage and abortion. Both findings were confirmed both *in vitro* an *in vivo*. On the other hand, the low-virulence isolate is quickly recognized and controlled by the placental immune responses, which along with the lower proliferation of this isolate, mainly in caruncular cells, suggest a different mechanism to cross the placental barrier. Modulation of the ECM and the hijacking of the immune cells may be the strategies used by this parasite in order to be transmitted avoiding placental damage. As previously suggested, when the parasite is transmitted from the dam to the offspring, a low virulence could be selected in order to preserve the success of transmission. Therefore, a correlation between low virulence and vertical transmission could be established (Rojo-Montejo *et al.*, 2009b).Trying to go deeper in the pathogenesis of the bovine neosporosis, comparison between viable and non-viable foetuses from Nc-Spain7-infected animals showed that hardly placental and foetal burden and lesions would explain the foetal death by themselves, and an implication of the maternal and foetal immune responses in the outcome of the infection was suggested. However, this experiment was not designed to elucidate the cause of the abortion and a closer monitoring of the foetus and sequential sampling and culling would be necessary.

In vitro results were confirmed *in vivo* proving the validity of both models for investigating the processes implicated in the lytic cycle of *N. caninum* tachyzoites and the early infection dynamics in the bovine placenta as well as for the study of the immune responses and ECM modulation induced in both cell lines after infection with two isolates of different virulence. These studies have revealed interesting insights into likely mechanisms involved in specific phenotypic traits and virulence in *N. caninum* and lay the foundations for further investigations.

CHAPTER VI

CONCLUSIONS

CONCLUSIONES

Objective 1: Characterization of parasite interaction between high- and low-virulence isolates of *N. caninum* and bovine placental target cells *in vitro*.

First. Bovine trophoblast and bovine caruncular epithelial cell lines are good models for exploring the pathways of *N. caninum* infection during transplacental transmission in the second term of pregnancy. Concretely, proliferation and processes implicated in the lytic cycle of *N. caninum* in the bovine placenta and immune responses induced in both cell lines after infection can be investigated using this model.

Sub-objective 1.1: Characterization of the lytic cycle of high- and low-virulence isolates of *N. caninum* in bovine placental target cells *in vitro*.

First. Parasite invasion, growth and egression mechanisms show clear differences between bovine trophoblast and caruncular cell lines which suggest that bovine caruncular cells act as a barrier in the bovine placenta, limiting the proliferation of *N. caninum* whereas bovine trophoblast cells act as a niche for parasite multiplication. Caruncular cells restrict *N. caninum* infection at two critical stages of the parasite lytic cycle: at the point of parasite adhesion to the host cell and intracellular replication. Despite the role as a barrier, an early egression mechanism described in caruncular cells and high proliferation in trophoblasts indicate that *N. caninum* is able to cross the caruncular layer of the bovine placenta facilitating rapid transmission of the parasite to the progeny.

Second. The highly virulent isolate Nc-Spain7 shows higher invasion and infection rates as well as higher proliferation than the low-virulence isolate Nc-Spain1H in bovine placental target cells, especially in trophoblast cells, that are one of the bases of their differences in virulence.

Sub-objective 1.2: *In vitro* interaction between *N. caninum* and the placental target cells from an immunological level.

First. Placental cells participate in the innate immune response against *N. caninum* at the maternal-foetal interface without any extra stimulus but the parasite, *via* a rapid pro-inflammatory response characterized by the overexpression of pro-inflammatory IL-8 and TNF-α and the downregulation of regulatory TGF-β1 and IL-6.

Second. Bovine trophoblast and caruncular cells are activated early after infection with the low-virulence isolate Nc-Spain1H inducing higher levels of pro-inflammatory TNF-α, which might lead to a better control of this isolate by the immune response. However, Nc-Spain7 does not activate TLR-2 either in trophoblast nor caruncular cells, indicating an evasion mechanism of the immune response by this isolate.

Objective 2: Study of the early infection in a pregnant bovine model inoculated with high- and low-virulence isolates of *N. caninum* at mid-gestation.

First. Different host-parasite interaction patterns are observed in the bovine placenta with high- and low-virulence isolates of *N. caninum* during early stages of infection at mid-gestation, suggesting the existence of different evolutionary adaptative strategies used by this parasite for transmission to the offspring.

Sub-objective 2.1: Early *N. caninum* infection dynamics in pregnant heifers after inoculation at mid-gestation with high- and low-virulence isolates.

First. The clinical consequences of *N. caninum* infection at mid-gestation are dependent on the isolate implicated in the infection. Nc-Spain7 tachyzoites are detected earlier than Nc-Spain1H tachyzoites in the placenta (at 10 days post-infection), replicating and leading to lesion development, transmission to the foetus and foetal death, whereas the low virulent isolate Nc-Spain1H showed a lower and delayed dissemination in the placental tissues without lesions development nor transmission during the first 20 days post-infection. These findings may be related to higher proliferation abilities of the high-virulence isolate Nc-Spain7.

Sub-objective 2.2: Placental immune response and extracellular matrix organization during the early stages of *N. caninum* infection in pregnant heifers inoculated with high- and low-virulence isolates at mid-gestation.

First. Modulation of the placental immune response at early stages of infection in pregnant cattle inoculated at mid-gestation is dependent on the isolate and the time post-infection.

At 10 dpi, the low-virulence isolate Nc-Spain1H triggers activation of classical PRRs and a general increase of the local immune responses, even though its presence is not detected in the placenta at this time, which may contribute to the control of this isolate. Contrary, the high-virulence isolate Nc-Spain7 is not recognized by the classical PRRs in the placenta until 20 dpi, which may indicate the existence of an evasion mechanism that may favour its multiplication and dissemination to the foetus. Detection of Nc-Spain7 when lesions are already delevoped might lead to exacerbate Th1 immune responses in an attempt to control the infection, which may contribute to the foetal death.

Second. Despite the lack of classical PRRs activation at 10 dpi, the high virulent isolate induces certain upregulation of inflammatory responses and infiltration of immune cells in the placenta at this time, suggesting the activation of other alternatives routes of the immune responses.

Third. A different mechanism of transmission between high and low virulent isolates is suggested. Extracellular matrix seems to be an important modulation point for Nc-Spain1H, which may use this mechanism to cross the placental barrier, maintaining the homeostasis of the placenta and avoiding foetal death. On the other hand, Nc-Spain7 would exploit its intrinsic invasion and replication abilities as well as an early evasion of the placental immune responses in order to arrive to the foetus, leading to placental damage, alteration of the local Th1/Th2 balance, with exacerbated immune responses in the placenta and, finally, to foetal death.

Fourth. An exacerbated pro-inflammatory immune response at the placenta, unbalanced in favour of Th1 responses, and with higher expression of abortifacient iNOS, TNF-α and IL-8 and wider inflammatory infiltrate in placentomes from animals carrying non-viable foetuses, together with a decrease of TGF-β1 may contribute to the foetal death. In addition, a profound alteration of the extracellular matrix, with decreased expression of MMP-2 and TIMP-2 and destruction of collagen, fibronectin and vimentin, and the lack of activation of tissue repair processes could contribute to the abortion mechanism.

Objetivo 1: Caracterización de la interacción parásito-hospedador entre aislados de alta y baja virulencia de *N. caninum* y células diana de la placenta bovina *in vitro*.

Primera. Las líneas celulares de trofoblasto bovino y de células epiteliales carunculares bovinas son buenos modelos para explorar las rutas de infección por *N. caninum* durante la transmisión transplacentaria en el segundo trimestre de la gestación. Concretamente, la proliferación y los procesos implicados en el ciclo lítico de *N. caninum* en la placenta bovina, así como las respuestas inmunitarias inducidas en ambas líneas celulares después de la infección pueden investigarse utilizando este modelo.

Subobjetivo 1.1: Caracterización del ciclo lítico de aislados de alta y baja virulencia de *N. caninum* en células diana de la placenta bovina *in vitro*.

Primera. Los mecanismos de invasión, proliferación y egresión del parásito muestran claras diferencias entre las células del trofoblasto bovino y las células carunculares. Los resultados sugieren que las células carunculares actúan como una barrera en la placenta bovina, limitando la proliferación de *N. caninum*, mientras que las células del trofoblasto actúan como un nicho para la multiplicación del parásito. Las células de la carúncula limitan la infección por *N. caninum* en dos etapas críticas del ciclo lítico del parásito: en el punto de adhesión del parásito a la célula hospedadora y en la replicación intracelular. A pesar del papel de barrera, un mecanismo de egresión temprana descrito en las células carunculares y la alta proliferación del parásito en las células del trofoblasto indican que *N. caninum* es capaz de atravesar la capa caruncular de la placenta bovina, facilitando la rápida transmisión del parásito a la descendencia.

Segunda. El aislado de alta virulencia Nc-Spain7 presenta mayores tasas invasión e infección, así como una mayor proliferación que el aislado de baja virulencia Nc-Spain1H en las células diana de la placenta bovina, especialmente en las células del trofoblasto, lo que supone una de las bases de sus diferencias de virulencia.

Subobjetivo 1.2: Interacción *in vitro* entre *N. caninum* y las células diana de la placenta bovina a nivel inmunológico.

Primera. Las células placentarias participan en la respuesta inmunitaria innata frente a *N. caninum* en la interfaz materno-fetal sin necesidad de ningún estímulo adicional excepto el parásito, a través de una rápida respuesta proinflamatoria caracterizada por un aumento en la expresión de las citoquinas proinflamatorias IL-8 y TNF-α y la disminución de la citoquina reguladora TGF-β1 y la IL-6.

Segunda. Las células del trofoblasto bovino y las células carunculares se activan poco después de la infección con el aislado de baja virulencia Nc-Spain1H, induciendo niveles más altos de la citoquina proinflamatoria TNF-α, lo que podría conducir a un mejor control de este aislado por parte de la respuesta inmunitaria. Sin embargo, el aislado de alta virulencia Nc-Spain7 no activa el receptor TLR-2 ni en las células de trofoblasto ni en las carunculares, lo que indica un mecanismo de evasión de la respuesta inmunitaria de este aislado.

Objetivo 2: Estudio de la infección temprana en un modelo bovino gestante inoculado con aislados de alta y baja virulencia de *N. caninum* en el segundo tercio de la gestación.

Primera. Se observan diferentes patrones de interacción parásito-hospedador en la placenta bovina con aislados de alta y baja virulencia de *N. caninum* durante la infección temprana en el segundo tercio de la gestación, lo que sugiere la existencia de diferentes estrategias evolutivas de adaptación utilizadas por este parásito para la transmisión a la descendencia.

Subobjetivo 2.1: Dinámica de la infección temprana en novillas gestantes tras la inoculación de aislados de alta y baja virulencia de *N. caninum* en el segundo tercio de la gestación.

Primera. Las consecuencias clínicas de la infección por *N. caninum* en el segundo tercio de la gestación dependen del aislado implicado en la infección. El aislado Nc-Spain7 se detecta antes en la placenta que el aislado Nc-Spain1H, replicándose y provocando el desarrollo de lesiones, la transmisión al feto y la muerte fetal. El aislado de baja virulencia Nc-Spain1H mostró una diseminación más baja y tardía en los tejidos placentarios sin que se produjeran lesiones ni se transmitiera durante los primeros 20 días tras la infección. Estos hallazgos pueden estar relacionados con la mayor capacidad de proliferación del aislado de alta virulencia Nc-Spain7 en la placenta bovina.

Subobjetivo 2.2: Respuesta inmunitaria en la placenta y organización de la matriz extracelular durante las primeras etapas de la infección con aislados de alta y baja virulencia de *N. caninum* en novillas gestantes inoculadas en el segundo tercio de la gestación.

Primera. La modulación de la respuesta inmunitaria de la placenta en las primeras etapas de la infección en novillas gestantes inoculadas en el segundo tercio de la gestación depende del aislado y del momento estudiado tras la infección.

A los 10 días tras la infección, el aislado de baja virulencia Nc-Spain1H desencadena la activación de los PRRs clásicos y un aumento general de la respuesta inmunitaria local que puede contribuir al control de la infección, a pesar de no ser detectado en la placenta en dicho momento. Por el contrario, el aislado de alta virulencia Nc-Spain7 no es reconocido por los PRRs clásicos en la placenta hasta los 20 dpi, lo que puede indicar la existencia de un mecanismo de evasión que favorece su multiplicación y diseminación al feto. La detección del aislado Nc-Spain7 por parte de la respuesta inmunitaria de la placenta cuando las lesiones ya se han producido podría llevar a una exacerbación de la respuesta de tipo Th1 en un intento de controlar la infección, contribuyendo a la muerte fetal.

Segunda. A pesar de la falta de activación de los PRRs clásicos a los 10 días post-infección, el aislado de alta virulencia induce un cierto aumento de la expresión de la respuesta inflamatoria, así como la infiltración de las células inmunitarias en la placenta, lo que sugiere la activación de otras rutas alternativas de la respuesta inmunitaria.

Tercera. Se sugiere un mecanismo de transmisión distinto entre aislados de alta y baja virulencia. La matriz extracelular parece ser un importante punto de modulación para el aislado de baja virulencia Nc-Spain1H, que puede utilizar este mecanismo para cruzar la barrera placentaria, manteniendo la homeostasis de la placenta y evitando la muerte fetal. Por otro lado, el aislado de alta virulencia Nc-Spain7 aprovecharía sus capacidades intrínsecas de invasión y proliferación,

así como la evasión temprana de la respuesta inmunitaria de la placenta para llegar al feto, lo que provocaría lesiones en la placenta, la alteración del equilibrio local de las respuestas Th1/Th2, con una respuesta inmunitaria exacerbada en la placenta y, finalmente, la muerte fetal.

Cuarta. Una respuesta inmunitaria proinflamatoria exacerbada en la placenta, con desequilibrio a favor de la respuesta de tipo Th1, mayor expresión de citoquinas y enzimas abortivas como la iNOS, el TNF-α y la IL-8 y un infiltrado inflamatorio más amplio en las placentas de las novillas con fetos no viables, junto con una disminución del TGF-β1 pueden contribuir a la muerte fetal. Además, la profunda alteración de la matriz extracelular, con disminución de la expresión de la MMP-2 y el TIMP-2 y la destrucción de colágeno, fibronectina y vimentina, así como la falta de activación de los procesos de reparación tisular podrían contribuir al mecanismo del aborto.

CHAPTER VII

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APPENDIX I

ORIGINAL MANUSCRIPTS

MANUSCRITOS ORIGINALES

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Differential susceptibility of bovine caruncular and trophoblast cell lines to infection with high and low virulence isolates of *Neospora caninum*

Laura Jiménez-Pelayo[1†], Marta García-Sánchez[1†], Javier Regidor-Cerrillo[1], Pilar Horcajo[1], Esther Collantes-Fernández[1], Mercedes Gómez-Bautista[1], Nina Hambruch[2], Christiane Pfarrer[2] and Luis Miguel Ortega-Mora[1*]

Abstract

Background: *Neospora caninum*, one of the main causes of abortion in cattle, is very effective at crossing the placental barrier and placental damage is crucial in the pathogenesis of abortion. Bovine trophoblast and caruncular cell layers are key cellular components in the maternal-foetal interface in placentomes, playing a fundamental role in placental functionality.

Methods: We studied tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence *N. caninum* isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells. The parasite invasion rate (pInvR) and the cell infection rate (cInfR) were determined by immunostaining plaque assay at different time points and multiplicities of infection (MOIs), respectively. In addition, tachyzoite growth kinetics were investigated using real-time PCR (qPCR) analysis and immunostaining plaque assay at different times.

Results: *Neospora caninum* invaded and proliferated in both cell lines. The pInvR was higher in F3 compared to BCEC-1 cells for the Nc-Spain7 isolate ($P < 0.05$), and higher for the Nc-Spain7 than the Nc-Spain1H in F3 cells ($P < 0.01$). The cInfR was also higher in F3 cells than in BCEC-1 cells for both isolates ($P < 0.0001$), and the cInfR for the Nc-Spain7 isolate was higher than for the Nc-Spain1H isolate in both cell lines ($P < 0.05$). Tachyzoite growth kinetics showed tachyzoite exponential growth until egress at 58 hpi for both isolates in F3, whereas Nc-Spain1H showed a non-exponential growth pattern in BCEC-1. Asynchronous egress of both isolates was observed from 22 h post-infection onwards in BCEC-1. In addition, the tachyzoite yield (TY_{58h}) was higher in F3 than in BCEC-1 infected by both isolates ($P < 0.0001$), highlighting better replication abilities of both parasites in F3. Nc-Spain7 showed shorter doubling times and higher TY_{58h} compared to Nc-Spain1H in F3 cells; adhesion, invasion and proliferation mechanisms were very similar for both isolates in BCEC-1.

Conclusions: Our results indicate a highly similar behavior of high- and low-virulence isolates in their interactions with maternal caruncular cells and suggest an important role of foetal trophoblasts in the pathogenesis of *N. caninum* infection.

Keywords: *Neospora caninum*, Cattle, Isolates, Virulence, Placenta, Trophoblast, Caruncle, Adhesion, Invasion, Proliferation

* Correspondence: luis.ortega@ucm.es
†Equal contributors
[1]SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
Full list of author information is available at the end of the article

Background

Neospora caninum is an apicomplexan protozoan parasite, phylogenetically related to *Toxoplasma gondii*. This parasite is considered a major cause of reproductive failure in cattle worldwide [1–3], resulting in great economic losses [4]. Infection in cattle may occur through horizontal transmission, when cattle ingest sporulated oocysts shed by a canid definitive host, or by endogenous congenital transmission, from a persistently infected dam to a foetus [5]. Oral infection or recrudescence in a pregnant cow can result in abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf [1].

Neospora caninum is one of the most efficiently transplacentally-transmitted organisms in cattle [5]. During natural infections, invasion of the placenta, proliferation and dissemination to the foetus are crucial events in the pathogenesis of bovine neosporosis and are related to the interactions of tachyzoites with host cells and its relationship with the local immune response at the maternal-foetal interface [6]. In vivo studies demonstrated that *N. caninum* is able to infect the maternal caruncular septum before crossing to the foetal placental villus [7, 8]. Despite the fact that the placenta is directly involved in the pathogenesis of the disease [9, 10], the mechanisms by which *N. caninum* infects the placenta and reaches the fetus are poorly understood [11]. One reason could be the placental diversity [12], which makes an extrapolation of findings from one species to the other difficult. To date, only one limited in vitro study investigating the potential involvement of bovine trophoblast in *N. caninum* infection has been published [13]. In addition, no information is available regarding in vitro infection in bovine caruncular epithelial cells and the role of placental cell layers in vertical transmission.

In addition, a key question in bovine neosporosis is the influence of the parasite intra-specific variability on the outcome of infection. The lytic cycle of *N. caninum* and other apicomplexan parasites comprises the processes of invasion, adaptation to intracellular conditions, proliferation, and egress from host cells [6, 14, 15]. This sequence of events is required for parasite survival and propagation in the course of animal infection. Our previous findings demonstrated that *N. caninum* isolates of bovine or canine origin show a large biological diversity, despite being genetically similar [16]. Moreover, differences found in the events of the lytic cycle among several *N. caninum* isolates in vitro are correlated with differences observed in virulence and vertical transmission in animal models [16, 17]. Specifically, pregnant heifers inoculated at day 70 of gestation with the low-virulence isolate Nc-Spain1H spared the foetus [18], whereas foetal death occurred in all inoculated cattle with the highly virulent isolate Nc-Spain7 [19, 20].

There is no information concerning the kinetics of events in the placenta that lead to an understanding of how the parasite actually reaches the foetal tissues. The influence of biological variability of the isolate on placental damage is also poorly understood. The cow possesses a cotyledonary [21] and synepitheliochorial placenta [22], where foetal cotyledons interdigitate with maternal caruncles to form placentomes [23–25]. The trophoblast (epithelial surface of the foetal cotyledons) consists of uninucleated and binucleated cells. Binucleated cells are responsible for a "restricted" trophoblast invasion [26], playing an important role in embryo implantation and successful pregnancy outcomes. Caruncular epithelial cells form a polarized barrier that the parasite encounters before reaching and multiplying in foetal tissues. Hence, the aim of this study was to investigate the interaction of two isolates of *N. caninum* with maternal and foetal bovine target cells. Here, we studied tachyzoite adhesion, invasion, proliferation and egress of high- (Nc-Spain7) and low- (Nc-Spain1H) virulence isolates in established cultures of bovine caruncular epithelial (BCEC-1) and trophoblast (F3) cells. Since BCEC-1 and F3 cells conserve some of the properties from their tissues of origin [24, 27, 28], they are a useful tool to evaluate critical factors involved in placental pathogenesis, such as the mechanisms used by *N. caninum* to cross the placental barriers.

Methods

Parasites and cell cultures

Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy, congenitally infected calves [29, 30] and extensively characterized using in vitro, murine and bovine models [16, 18, 20, 29, 31, 32]. Tachyzoites were routinely maintained in a monolayer culture of the MARC-145 monkey kidney cell line as described previously [16]. The *N. caninum* isolates used in this study were subjected to a limited number of culture passages (from 8 to 15) to ensure the maintenance of their in vivo biological behaviour and avoid their adaptation to the host cells [33].

A bovine trophoblast cell line F3 [28] and a bovine caruncular cell line BCEC-1 [23] were isolated from two BVD-free, pregnant cattle (*Bos taurus*) with an estimated gestational age of 5 and 4 months, respectively. Cells were grown as indicated by Hambruch et al. [28]. Briefly, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 containing 10% foetal calf serum (FCS) checked for the absence of specific IgG against *N. caninum* by IFAT, 100 IU/ml Penicillin, 100 mg/ml Streptomycin and 2 mM Glutamine. All experiments were carried out with cells below passage 27, when both cell lines maintained their morphological and functional features [24, 27, 28].

Tachyzoites used for in vitro assays were recovered from 2.5–3 day growth cultures of MARC-145, when the majority of the parasites were still intracellular, and purified using Disposable PD-10 Desalting Columns (G.E. Healthcare, Buckinghamshire, UK) as previously described [16]. Tachyzoite viability was checked by trypan blue exclusion. F3 and BCEC-1 cell monolayers were inoculated within 1 hour of tachyzoite collection from flasks. All in vitro experiments in F3 and BCEC-1 cell lines were assayed in quadruplicate, and two independent experiments were carried out.

Parasite invasion rate

Parasite invasion rate (pInvR) was defined as the number of tachyzoites invading the host cell at different time-points (hours) post-infection (hpi) and were determined following the methodology described in Dellarupe et al. [17] with minimal modifications. In order to obtain a confluent monolayer of F3 and BCEC-1, cells were seeded with 2×10^5 and 3×10^5 cells per well, respectively. Different density of both cell types were used because F3 cells are bigger than BCEC-1 cells and formed a monolayer composed of polygonal cells while as BCEC-1 are smaller and they tended to form colonies and did not spread out the entire surface of the well. A total of 100 purified tachyzoites of each isolate were added to 24-well culture plates. Cultures were washed three times with phosphate buffered saline (PBS) at different time points (1, 2, 4, 6 and 8 hpi) for removing non-adhered/non-invading tachyzoites. Unwashed cultures were also included in the study. All plates were fixed at 48 hpi, and the pInvR was determined using single immunofluorescence staining as described below. To determine the pInvR, events (medium and large parasitophorous vacuoles) present in each well were counted using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 200×. The pInvR at 1, 2, 4, 6 and 8 hpi ($pInvR_{1h}$, $pInvR_{2h}$, $pInvR_{4h}$, $pInvR_{6h}$, $pInvR_{8h}$, respectively) was determined as the number of events per well in cell monolayers washed at different time points, and the total parasite invasion rate ($pInvR_T$) was determined as the number of events per well in unwashed cultures.

Cell infection rate

Multiplicity of infection (MOI) was defined as the ratio of the number of tachyzoites added to a known number of cells in a culture. Cell infection rate (cInfR) was defined as the percentage of cells infected using different MOIs (1, 2, 4, 6, 8 and 10). Cells were cultured in 24-well plates at concentration of 2×10^5 and 3×10^5 cells per well for F3 and BCEC-1 cells, respectively. Infected cells were washed 3 times with PBS after 4 hpi to facilitate the synchronization of the cultures. Finally, cells were fixed at 48 hpi and stained using single immunofluorescence staining as described below.

The overall number of cells, the number of infected cells and the number of cells containing more than one vacuole (multi-infected cells) were counted in 10 arbitrarily selected fields using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 200×. Counting of events was carried out on images taken with three different filters (white light for discrimination of cell limits, blue-DAPI for visualization of the nuclei and red-Alexa 594 for examination of the tachyzoites) using a Nikon DSL1 camera (Chiyoda, TYO, Japan) and overlaid using Photoshop® software (Adobe Systems Incorporated, Mountain View, CA, USA). A mean value of 50 cells was counted in each field.

Adhesion-invasion assay

An adhesion-invasion assay was performed in F3 and BCEC-1 cultures seeded at concentration of 2×10^5 and 3×10^5 cells per well, respectively. Cells were infected at a MOI of 2, and cultures were washed with PBS at 4 hpi to remove non-adherent extracellular tachyzoites. Cultures were immediately fixed and double immunofluorescence staining was carried out following the protocol described below. Adhered extracellular tachyzoites (green- and red-stained) and intracellular tachyzoites (red-stained only) were counted using a fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan) at a magnification of 400×. A total of 1000 tachyzoites was counted in each coverslip. The percentage of intracellular tachyzoites (red-stained) respect to the total number of tachyzoites (intracellular and extracellular adhered tachyzoites) (green-stained) at 4 hpi was calculated.

Intracellular proliferation assays: Proliferation kinetics, doubling time and tachyzoite yield determinations

Proliferation kinetics of Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cells were determined by quantifying the number of tachyzoites at specific times (4, 10, 22, 34, 46, 58, 70 and 82 hpi) by real-time PCR (qPCR). Cells were cultured and infected as indicated above using a MOI of 2. Cultures were washed at 4 hpi and subsequently maintained at 37 °C in 5% CO_2. The samples were collected adding 200 μl of PBS, 180 μl of lysis buffer and 20 μl of proteinase K (Qiagen, Hilden, Germany) to each well at 4, 10, 22, 34, 46, 58, 70 and 82 hpi, transferred to a microcentrifuge tube and frozen at -80 °C prior to DNA extraction.

In parallel, replicates of cell cultures in coverslips were infected as described above and were labelled using double-inmunostaining to study microscopically the proliferation kinetics of both isolates in F3 and BCEC-1 cells. Three coverslips were photographed for each

condition using an inverted fluorescence microscope (Nikon Eclipse TE 200, Chiyoda, TYO, Japan).

The doubling time (T_d) was defined as the period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding the lag phase (period without parasite multiplication) and the egress phase [16]. The T_d was determined by applying non-linear regression analysis and an exponential growth equation using GraphPad (San Diego, CA, USA). We represented T_d for each isolate and each cell line as the average value obtained from all the determinations that revealed a linear regression, $R^2 \geq 0.95$.

The tachyzoite yield (TY_{58h}) was defined as the average value of the number of tachyzoites quantified by qPCR at 58 hpi for each isolate and cell line.

Immunofluorescence staining

Single immunofluorescence staining was carried out as specified previously [17] with minimal variations. Parasites in fixed cultures were stained using hyperimmune rabbit antiserum directed against *N. caninum* tachyzoites (1:1000) as a primary antibody and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 594 (red, Thermo Fisher Scientific, Waltham, MA, USA) as a secondary antibody. The nuclei were stained by washing the cells with a solution of 1:5000 DAPI in PBS.

Double immunofluorescence staining was carried out following the protocol described by Regidor-Cerrillo et al. [16] with minimal modifications. Fixed plates were treated with 3% BSA in PBS for 30 min at 25 °C to block unspecific antibody binding and autofluorescence. Then, cultures were treated with a 1:1000 dilution of anti-tachyzoite hyperimmune rabbit antiserum and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 488 (green, Thermo Fisher Scientific, Waltham, MA, USA). After this step, only extracellular tachyzoites were labelled in green. After the first staining, cells were permeabilized using a solution of 0.25% Triton 100X in PBS 0.3% BSA (30 min, 37 °C). Later, cultures were treated again with a dilution of anti-tachyzoite hyperimmune rabbit antiserum as primary antibody (1:1000) and a 1:1000 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor® 594 as secondary antibody (red, Thermo Fisher Scientific, Waltham, MA, USA). Therefore, intracellular tachyzoites were labelled only in red, while extracellular tachyzoytes resulted labelled in green and in red. The nuclei were stained by washing the cells with a solution of 1:5000 DAPI in PBS and the coverslips were embedded in Fluoroprep (BioMerieux, Marcy-l'Étoile, France).

DNA extraction and real-time PCR

Genomic DNA was extracted from cellular samples using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was eluted in a volume of 60 μl of molecular-grade water. Concentrations of DNA were determined for each sample using a nanophotometer (Nanophotomer®, Implen GmbH, Munich, Germany) and samples were diluted 1:4 in molecular-grade water. Quantification of *N. caninum* DNA was performed by real-time PCR using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The Nc-5 region was targeted as described elsewhere [34]. Five μl of diluted DNA from each sample were used for the qPCR assays. The number of *N. caninum* tachyzoites was determined by interpolating the C_t values (cycle threshold value, which represents the fractional cycle number reflecting a positive PCR result) on a standard curve. The standard curve was designed for the quantification of 10^{-1}–10^4 tachyzoites according to Regidor-Cerrillo et al. [16]. To normalize the quantification of the parasites in each sample, a bovine β-actin standard curve was designed (from 64 ng of DNA per μl to 0.2 ng per μl). The results were expressed as the relation between amounts of parasite DNA and cell DNA ($R^2 \geq 0.99$; slope values varied from -3.67 to -3.13).

Statistical analysis

The parametric one-way ANOVA test, followed by a Tukey's multiple comparisons test, was performed to investigate the influence of time on the pInvR and MOI in the cInfR, and the two-way ANOVA test, followed by a Tukey's multiple comparisons test, was performed to study the influence of the parasite isolate and the cell type on the pInvR and cInfR. A Chi-square test was carried out to investigate the differences in the percentages of intracellular tachyzoites at 4 hpi in both target cell types. Bonferroni correction was used to eliminate error associated with making multiple comparisons. Statistical significance was established as $P < 0.05$. Differences that showed P-values ≥ 0.05 and < 0.1 were considered to be trending towards statistical significance. GraphPad Prism 5 v.5.01 (San Diego, CA, USA) software was used to perform all statistical analyses and graphical illustrations.

Results

Parasite invasion rate (pInvR)

To investigate the impact of the placental cell type in parasite invasion, the pInvR was evaluated in trophoblasts and caruncular cells at different time points post-infection (1, 2, 4, 6 and 8 hpi). The pInvRs of the Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cells are shown in Fig. 1. The number of invaded tachyzoites for both isolates significantly increased until 4 hpi in both F3 (Nc-Spain7, ANOVA: $F_{(5,35)} = 11.87$, $P < 0.0001$; Nc-Spain1H, ANOVA: $F_{(5,35)} = 7.211$, $P < 0.0001$, followed by a Tukey's multiple comparisons test) and BCEC-1 cells (Nc-Spain7, ANOVA:

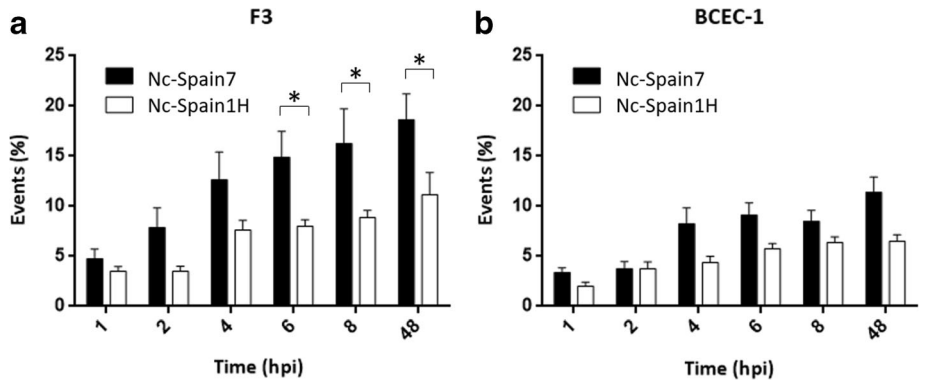

Fig. 1 Parasite invasion rates in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H isolates. Graphs represent parasite infection rates in F3 (**a**) and BCEC-1 cells (**b**) defined as the percentage of invaded tachyzoites (number of events per well) studied at different time points for Nc-Spain7 and Nc-Spain1H. Each column and error bar represents the mean and the SD of 4 replicates from 2 independent assays at the indicated sampling times. The total number of invaded tachyzoites was determined by single immunofluorescence staining of events (parasitophorous vacuoles and lysis plaques) followed by counting using an inverted fluorescence microscope. Significantly higher pInvRs were found in F3 cells compared to BCEC-1 cells infected with Nc-Spain7 ($P < 0.01$), whereas no differences were found in the pInvRs of F3 and BCEC-1 cells infected by Nc-Spain1H ($P > 0.05$). * represents significant differences between isolates

$F_{(5,35)} = 9.825$, $P < 0.0001$; Nc-Spain1H, ANOVA: $F_{(5,35)} = 9.156$, $P < 0.0001$, followed by a Tukey's multiple comparisons test). From 4 hpi onwards, significant differences were not observed.

Regarding the influence of the target cell type, a higher pInvR was observed in F3 cells compared to BCEC-1 cells from 6 hpi onwards for the Nc-Spain7 isolate (two-way ANOVA test: $F_{(3,168)} = 27.25$, $P < 0.0001$, followed by a Tukey's multiple comparisons test). No statistically significant differences were found when the pInvRs of Nc-Spain1H in both cell lines were investigated.

The influence of the parasite isolate on the invasion of bovine trophoblasts and caruncular cells was also investigated by comparison of the pInvRs between the Nc-Spain1H and Nc-Spain7 isolates, assayed at different times of infection. Nc-Spain7 showed a pInvR significantly higher than Nc-Spain1H from 6 hpi onwards in F3 cells (two-way ANOVA test: $F_{(3,168)} = 27.25$, $P < 0.0001$ followed by a Tukey's multiple comparisons test) (Fig. 1a). However, no statistically significant differences in pInvR were found between isolates in BCEC-1 cells (Fig. 1b).

Cell infection rate (cInfR)

The percentage of infected cells (cInfR) and the percentage of multi-infected cells were evaluated at different MOIs.

The number of infected cells significantly increased with increasing MOIs in both cell lines F3 (Nc-Spain7, ANOVA: $F_{(5,42)} = 228.5$, $P < 0.0001$; Nc-Spain1H, ANOVA: $F_{(5,42)} = 273.4$, $P < 0.0001$, followed by a Tukey's multiple comparisons test) and BCEC-1 (Nc-Spain7, ANOVA: $F_{(5,42)} = 30.04$, $P < 0.0001$; Nc-Spain1H, ANOVA: $F_{(5,42)} = 42.60$, $P < 0.0001$, followed by a Tukey's multiple comparisons test). The cInfRs were higher in infected F3 than in BCEC-1 cells for both Nc-Spain7 and Nc-Spain1H isolates at the same MOI (Fig. 2a, b) (two-way ANOVA test: $F_{(3,168)} = 222.4$, $P < 0.0001$, followed by a Tukey's multiple comparisons test). The percentage of cells containing more than a single vacuole (Fig. 2c, d) was also higher in F3 than in BCEC-1 infected by both isolates (two-way ANOVA test: $F_{(3,168)} = 93.64$, $P < 0.0001$, followed by a Tukey's multiple comparisons test).

In addition, Nc-Spain7 showed a higher cInfR than Nc-Spain1H in both cell lines at different MOIs (two-way ANOVA test: $F_{(3,168)} = 222.4$, $P < 0.0001$, followed by a Tukey's multiple comparisons test) (Fig. 2a, b). We also observed that Nc-Spain7 showed a higher percentage of multi-infected cells than Nc-Spain1H in F3 cells at 6, 8 and 10 MOIs (two-way ANOVA test: $F_{(3,168)} = 93.64$, $P < 0.0001$, followed by a Tukey's multiple comparisons test) (Fig. 2c). However, no significant differences in the number of multi-infected cells were found between isolates in BCEC-1 cells (Fig. 2d).

Adhesion-invasion assay

In the light of the differences in pInvR and cInfR between both cells lines, an adhesion-invasion assay was performed to investigate whether these differences could be attributed to a different adhesion ability of the tachyzoites in these two cell lines, a different ability to penetrate in the cells or both (Fig. 3a). In this assay, non-adhered tachyzoites were eliminated in the washing step at 4 hpi before the fixation and extra- and intracellular adhered tachyzoites were counted. The percentage of intracellular tachyzoites respect to the total adhered intra- and extracellular tachyzoites was calculated.

Surprisingly, both isolates showed that almost 100% of adhered tachyzoites were intracellular at 4 hpi in BCEC-1 cells, whereas a minor percentage of intracellular

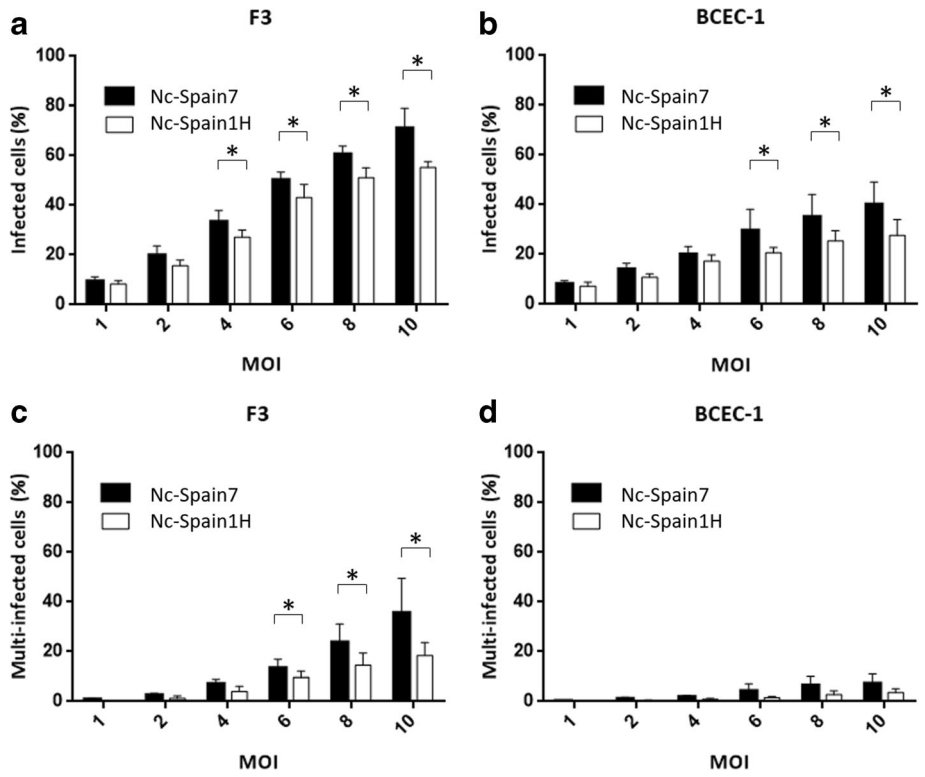

Fig. 2 Infection and multi-infection rates in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H isolates. Graphs represent the cell infection rates as the percentage of infected cells in F3 (**a**) and BCEC-1 cells (**b**) for both isolates and the percentage of cells with multi-infection (more than one parasitophorous vacuole) in F3 (**c**) and BCEC-1 cells (**d**). Each column and error bar represents the mean and the SD of 4 replicates from 2 independent assays using different MOIs. The total number of cells, the number of infected cells and the number of cells with multi-infection were determined by double immunofluorescence staining followed by counting using an inverted fluorescence microscope. The cInfRs were higher in F3 than in BCEC-1 cells infected by both isolates ($P < 0.0001$). The percentage of cells containing more than a single vacuole was also higher in F3 than in BCEC-1 cells infected by both isolates ($P < 0.05$). * represents significant differences between isolates

tachyzoites was observed in F3 cells, 88 and 69% for Nc-Spain7 and Nc-Spain1H, respectively. A significantly higher number of adhered tachyzoites from both isolates were internalized in BCEC-1 cells than in F3 cells at 4 hpi (Chi-square test: $\chi^2 = 287.6$, $df = 3$, $P < 0.0001$) (Fig. 3b).

Differences between isolates were not observed in BCEC-1, although the high-virulence isolate Nc-Spain7 showed a better ability to penetrate than the low-virulence isolate Nc-Spain1H in F3 cells (Fisher's exact test: $P < 0.0001$) (Fig. 3b).

Proliferation kinetics, doubling time comparisons and tachyzoite yield determination

An in vitro intracellular proliferation assay was carried out to study proliferation and egress events of the lytic cycle of *N. caninum* in trophoblast and caruncular cell cultures. Proliferation kinetics over time assessed by qPCR are represented in Fig. 4a, b. The growth curves of both isolates in F3 and the growth curve of Nc-Spain7 in BCEC-1 adjusted to exponential growth from 10 hpi to 70 hpi, whereas the growth curve of Nc-Spain1H in BCEC-1 did not adjust either to the exponential or linear growth mathematical model. Analysing the T_d, we observed a delay in the multiplication of *N. caninum* in BCEC-1 cells, with the average T_d value of Nc-Spain7 1.5-times higher in BCEC-1 cells (14.603 ± 1.428) than in F3 cells (9.425 ± 0.239) (one-way ANOVA: $F_{(2,21)} = 6.966$, $P = 0.0048$ followed by a Tukey's multiple comparisons test). The Nc-Spain1H isolate showed an average T_d value of 12.246 ± 0.893 in F3 cells. The T_d value for Nc-Spain1H in BCEC-1 could not be calculated due to the lack of exponential growth of Nc-Spain1H in BCEC-1. Nevertheless, no significant differences were found in the average T_d values for Nc-Spain7 and Nc-Spain1H isolates in F3 cells.

A microscopic examination of cultures fixed at different time points showed that the multiplication of Nc-Spain7 and Nc-Spain1H isolates began between 10 and 22 hpi. Differences in the parasitophorous vacuole size between both isolates were observed in F3 cells from 34 hpi onwards, with bigger vacuoles in Nc-Spain7 infected cells. However, no differences between the isolates were demonstrated by immunofluorescence in BCEC-1 cells. Between 58 and 82 hpi, asynchronous rupture of host cells and

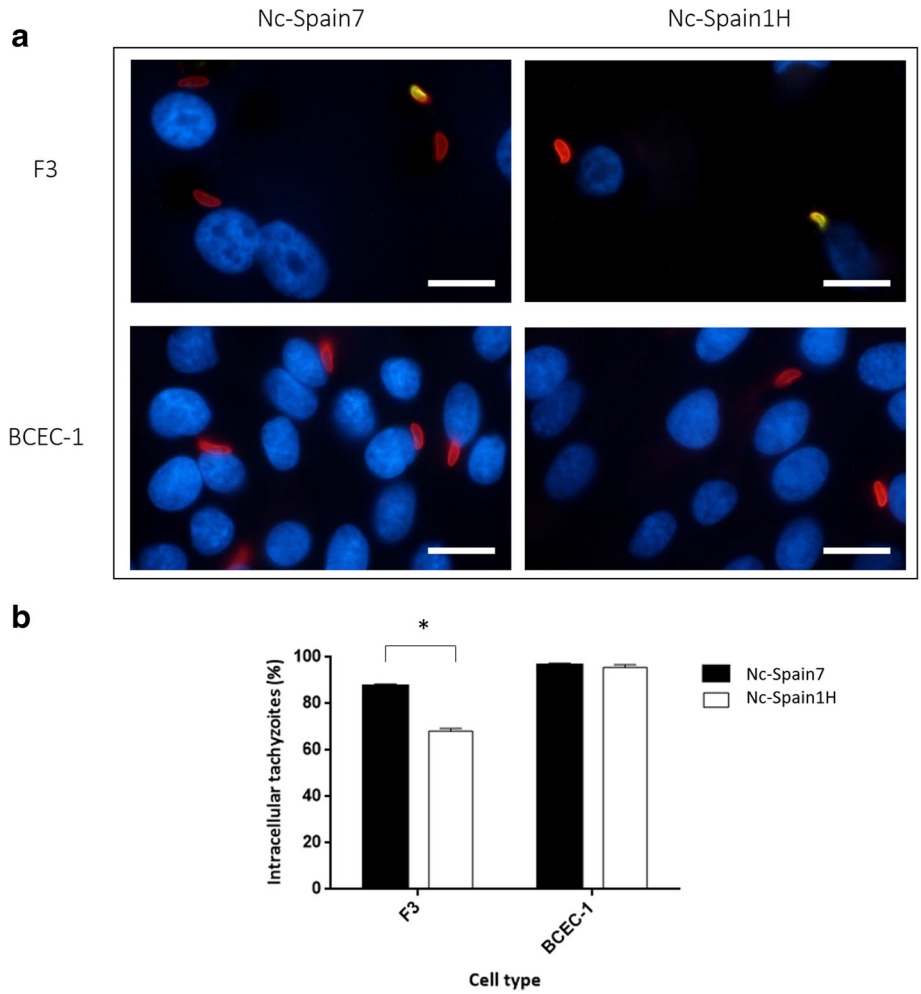

Fig. 3 Adhesion assay in F3 and BCEC-1 cells infected by Nc-Spain7 and Nc-Spain1H at 4 hpi. Double immunofluorescence staining was performed, and adhered extracellular tachyzoites were stained with Alexa Fluor® 488 (*green*) and Alexa Fluor® 594 (*red*), whereas intracellular tachyzoites were stained with Alexa Fluor® 594 (*red*). Nuclei were stained with DAPI (*blue*). Tachyzoites were counted in 10 arbitrarily selected fields, and the percentage of intracellular tachyzoites relative to the number of total adhered tachyzoites at 4 hpi was calculated. Representative images at a magnification of 1000× (**a**) show the adhesion assay performed in F3 and BCEC-1 cells infected with both isolates. The graph (**b**) represents the percentage of intracellular tachyzoites of Nc-Spain7 and Nc-Spain1H relative to the total number of intra- and extracellular tachyzoites adhered to F3 and BCEC-1 cells. Each column and error bar represents the mean and the SD of 4 replicates from 2 independent assays. BCEC-1 cells showed a significantly higher percentage of intracellular tachyzoites than F3 cells ($P < 0.0001$, Chi-square test). The percentage of intracellular tachyzoites for Nc-Spain7 (88%) was significantly higher than for Nc-Spain1H (69%) in F3 ($P < 0.0001$), whereas the percentage of intracellular tachyzoites of both isolates in BCEC-1 was the same (96%). * represents significant differences between isolates. *Scale-bars*: **a**, 40 μm

egress of the tachyzoites were observed in F3 cells. However, interestingly, an early egression of tachyzoites from 22 hpi onwards was observed in BCEC-1 (Fig. 4c).

The TY_{58h} was assessed to determine the number of tachyzoites produced during the same intracellular period after invasion, prior to complete tachyzoite egress from cell cultures (Fig. 4d). The TY_{58h} was 15-times higher in F3 cells than in BCEC-1 cells infected with Nc-Spain7, and 10-times higher in F3 cells infected with Nc-Spain1H. Comparing the isolates, Nc-Spain7 showed a higher TY_{58h} than Nc-Spain1H in F3 (one-way ANOVA: $F_{(3,28)} = 37.35$, $P < 0.0001$ followed by a Tukey's multiple comparisons test), whereas no differences in the TY_{58h} were found in BCEC-1.

The results obtained in the present work are summarized in Table 1.

Discussion

In the present study, we established for the first time a species- and organ-specific in vitro model for each of the host cell layers in the maternal-foetal interface of the bovine placenta to study *N. caninum* infection. To date, several in vitro studies have been carried out using established cell lines, such as Marc-145, HeLa, BeWo or

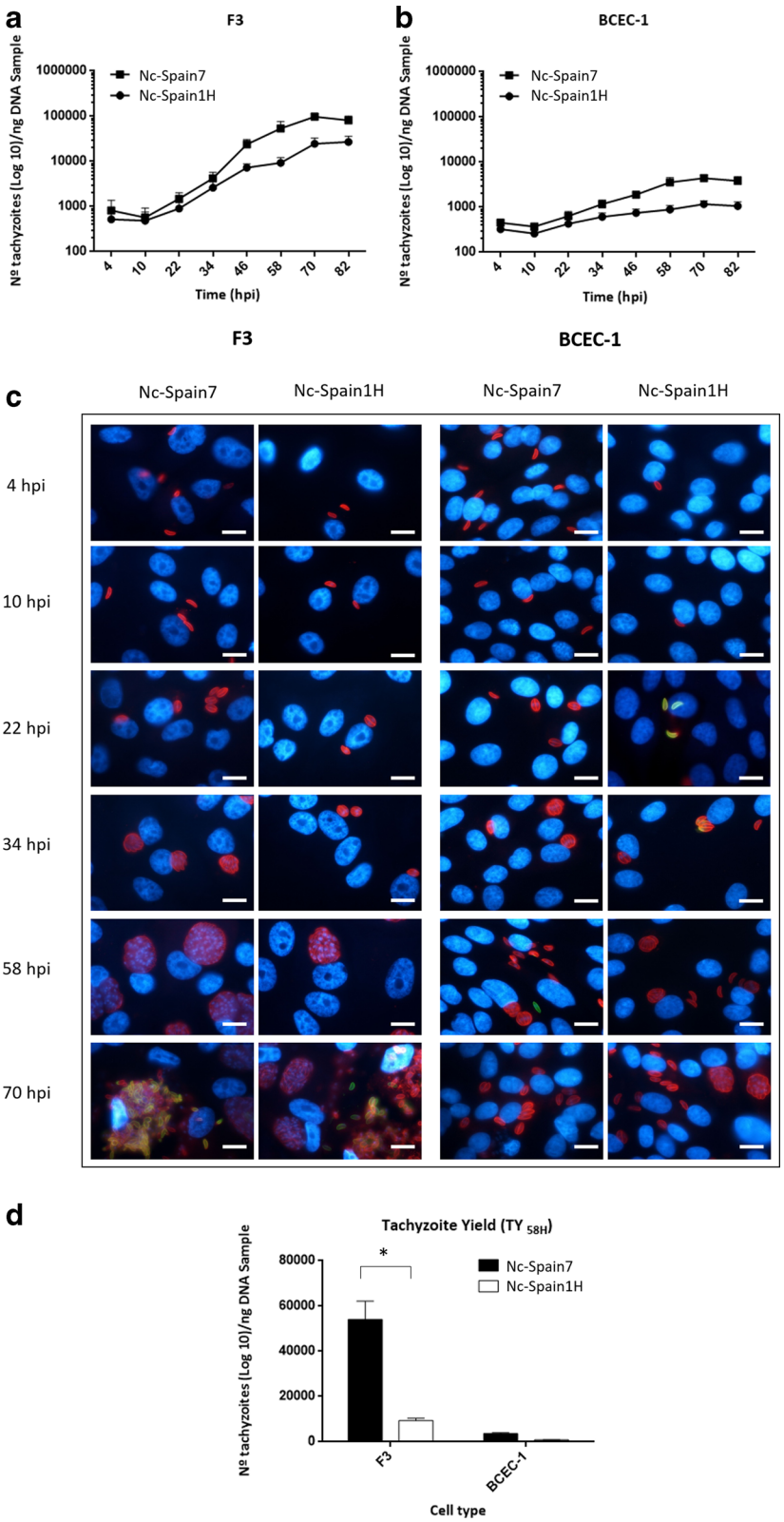

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Proliferation kinetics over time and tachyzoite yield at 58 hpi. Graphs (**a** and **b**) represent the average number of tachyzoites for each time-point for all individual experiments with an $R^2 > 0.95$, except for BCEC-1 cells infected by Nc-Spain1H, which showed a non-exponential growth pattern. Error bars indicate the SD. Representative images (**c**) show the proliferation kinetics over time of Nc-Spain7 and Nc-Spain1H isolates in F3 and BCEC-1 cultures. The bar graph (**d**) represents the tachyzoite yield at 58 hpi for Nc-Spain7 and Nc-Spain1H in F3 and BCEC-1 cells. Each column and error bar represents the mean and the SD of 4 replicates from 2 independent assays. The TY_{58h} was fifteen times higher in F3 cells than in BCEC-1 cells infected with Nc-Spain7, and ten times higher in F3 cells infected with Nc-Spain1H. Statistical differences were found in the TY_{58h} between isolates in F3 cells, with the TY_{58h} of Nc-Spain7 significantly higher than for Nc-Spain1H ($P < 0.0001$). * represents significant differences between isolates. *Scale-bars*: **c**, 10 μm

ovine trophoblast cells, to investigate the invasion and proliferation of different *N. caninum* isolates [16, 35, 36]. There is only one (limited) descriptive study concerning the interaction of the parasite with bovine trophoblast cells [13], and no data are available about the parasite's interaction with bovine caruncular cells. The interaction between *N. caninum* and these target cells has been studied in the present work, using two isolates of different virulence and the two cell lines that represent the maternal-foetal interface. The cell lines used in this work (bovine trophoblast cells, F3, and bovine caruncular epithelial cells, BCEC-1) were isolated from fifth- and fourth-month pregnant heifers, respectively, and they have maintained at least part of their morphological and functional characteristics [24, 27, 28]. Thus, they may be a useful tool to investigate the pathways of *N. caninum* infection during transplacental transmission during the second trimester of pregnancy when the majority of abortions caused by *N. caninum* occur [1]. Investigations were focused on the lytic cycle of the tachyzoites (host-cell invasion, proliferation and egress). The processes implicated in the lytic cycle of the parasite are essential for the invasion of host tissues, the distribution of the parasite through the organism and its distribution to the placenta. As a consequence, abortion or transplacental transmission may occur [1, 6]. Here, two *N. caninum* isolates with marked differences in virulence were able to establish themselves and multiply both in the maternal ephitelium of the caruncle and in the foetal trophoblast, although differences in the infection of both cell types were found.

Our results showed a lower infection rate, as well as a lower percentage of cells with multi-infection, for both parasite isolates in BCEC-1 cells relative to F3 cells. Therefore, *N. caninum* tachyzoites more efficiently infect trophoblast cells compared to bovine caruncular epithelial cells, meaning bovine trophoblast cells are more susceptible to *N. caninum* infection. In experimental infections, higher parasite burdens and more severe lesions have been found in the foetal part of the placenta [19, 20]. As previously demonstrated, BCEC-1 is an established cell line showing in vitro characteristics of a morphologically and functionally intact epithelial barrier with apical microvilli and junctional complexes (zonula occludens, zonula adherens and desmosomes) [23, 24] as described in vivo [37, 38]. This polarized barrier, with apical junctional complexes obliterating the paracellular space, establishes an effective paracellular barrier to diffusion of fluid and solutes, limiting the passage of foetal and maternal metabolites [39]. These characteristics may be hindering the paracellular passage of *N. caninum* across the epithelium. Foetal cells (F3) share many properties with maternal cells (BCEC-1), including apical microvilli and expression of the tight junctional zonula

Table 1 Summary of virulence traits as a function of cell type and *N. caninum* isolate

		pInvR[a]	cInfR[b]	% Multi-infection[c]	Invasion efficiency[d]	TY^{e}_{58H}	T_d^f
Cell type comparisons (F3 *vs* BCEC-1)	Nc-Spain7	+	++++	+	----	+	--
	Nc-Spain1H	NS[h]	++++	+	----	+	NC[g]
Isolate comparisons (Nc-Spain7 *vs* Nc-Spain1H)	F3	++	+	++++	++++	++	NS
	BCEC-1	NS	+	NS	NS	NS	NC[g]

[a]pInvR (parasite invasion rate): number of tachyzoites invading the host cell at different time points post-infection
[b]cInfR (cell infection rate): percentage of cells infected using different parasite doses
[c]% Multi-infection: percentage of cells containing more than one vacuole
[d]Invasion efficiency: results from adhesion-invasion assay, percentage of intracellular tachyzoites relative to the total number of tachyzoites at 4 hpi
[e]TY_{58H} (tachyzoite yield at 58 hpi): average number of tachyzoites quantified by qPCR at 58 hpi
[f]T_d (Doubling time): period of time required for a tachyzoite to duplicate during the exponential multiplication period, excluding lag and egress phases
[g]*NC* data not comparable. The T_d value for Nc-Spain1H isolate in BCEC-1 cells could not be calculated due to the lack of exponential growth of Nc-Spain1H in BCEC-1
[h]*NS* no significant differences
+/++/++++ indicate higher rates of each parameter assayed with a significance of $P < 0.05$, $P < 0.01$, $P < 0.0001$, respectively
-/--/---- indicate lower rates of each parameter assayed with a significance of $P < 0.05$, $P < 0.01$, $P < 0.0001$, respectively

occludens protein both in vivo [37, 38] and in vitro [28]; however, in contrast to the maternal BCEC-1 cells, mononuclear trophoblast cells have phagocytic phenotypes in vivo [40]. Also bovine F3 cells may form binucleated cells [28], which have phagocytic activity as has been previously described for trophoblast giant cells of various species [40–42]. The phagocytic activity of mono- and binucleate trophoblast cells may be mediating parasite passage to the foetus [13]. In fact, in a BALB/c mouse model infected with *T. gondii*, a higher frequency of infected placentas was observed at later stages of pregnancy, which has been correlated with a higher phagocytic efficiency of the placental tissues in this period [43]. Therefore, although differences in junctional complexes between both cell types should be investigated to evaluate their influence in parasite invasion, it seems probable that the phagocytic ability of trophoblast cells may partially explain the higher susceptibility to parasite invasion observed in these cells.

Noting the differences in the invasion and infection rates between both cell lines, an adhesion-invasion assay was performed in order to elucidate whether these differences could be associated with lower adhesion, penetration or both. Our results revealed that, contrary to expectations, both isolates, which had presented lower cell infection rate in BCEC-1 cells compared to F3 cells, showed a highly efficient invasion with almost 100% penetration of the adhered tachyzoites in BCEC-1 cells at 4 hpi. Thus, the lower invasion of *N. caninum* observed in BCEC-1 cells may be due to a lower ability to adhere or to a fragile adhesion to host-cell receptors. Tachyzoite adhesion occurs in two phases, as previously described [6]. The first step is the establishment of low-affinity contact between tachyzoites and the host-cell surface membrane, where surface antigens of *N. caninum* tachyzoites such as NcSAG1 and NcSRS2 are involved. Later, the actual adhesion process occurs via to microneme proteins (especially NcMIC3), which bind to host-cell surface chondroitin sulfates. Studying the differences in the type and abundance of superficial receptors responsible for the high-affinity interaction with tachyzoites between both cell lines could aid the understanding of the diminished adhesion of *N. caninum* in bovine caruncular cells.

Concerning the growth kinetics of *N. caninum* in foetal and maternal cells, our results showed a dramatically lower proliferation of both isolates in caruncular cells. Moreover, Nc-Spain1H did not adjust to an exponential growth in maternal cells. Differences observed between both cell types may be partially attributed to a different degree of maturation. While the foetus is not completely immunocompetent in the second trimester of gestation, maternal cells have immunocompetent abilities that may restrict the infection and proliferation of the parasite. In vivo studies have demonstrated the influence of the gestational stage on the outcome of *N. caninum* infection [44–47]. It is known that the survival of the foetus depends on the state of development of its immune system, as higher abortion rates, higher parasite burdens and more severe lesions were observed in foetal tissues when infection occurred in the first and second trimester of gestation [44, 46, 47]. On the other hand, the lower multiplication of *N. caninum* in the maternal side of the placenta supports the hypothesis that caruncles act as a barrier, limiting not only parasite infection via reduced adhesion but also its multiplication. In experimental infections, comparisons between cytokine mRNA levels in separated maternal and foetal placental tissues showed that maternal tissue was the major source of most cytokines [48] and had a major lymphocyte cell infiltration, particularly in the maternal caruncle [7, 49], which may indicate that the maternal immune system was actively responding to the parasite. Moreover, early egress was observed in caruncular cells, which could be employed by the parasite as an escape mechanism to facilitate the dissemination of the parasite to the foetal part of the placenta, which has been demonstrated in this work to be the parasite's preferential target cell, and, thus, allow vertical transmission of the parasite.

As mentioned above, the role of the parasite in the outcome of infection is also a determining factor. Processes involved in the lytic cycle, including parasite invasion and intracellular proliferation, are essential for the maintenance and multiplication of the parasite in vitro and for parasite survival and propagation in host tissues during the course of animal infection [1, 6]. In previous studies, several *N. caninum* isolates showed differences during the in vitro lytic cycle and more virulence than others in animal models, associated with higher abortion and transmission rates [16, 18, 20, 29, 31, 50]. In trophoblast cells, both isolates, described as "highly prolific" (Nc-Spain7) and "less prolific" (Nc-Spain1H) in previous studies [16], showed the same in vitro characteristics. In particular, the virulent isolate Nc-Spain7 demonstrated greater invasion, infection and proliferation rates than Nc-Spain1H in trophoblast cells. These differences may be explained by their biological diversity, as has been demonstrated in previous in vivo studies [16, 18, 20, 29, 32, 51, 52]. Nc-Spain7 showed a high neonatal mortality (95%) and vertical transmission rate (nearly 80%) in a pregnant BALB/c mouse model [31], as well as a percentage of abortion and vertical transmission as high as 100% in a bovine model [20, 53]. However, Nc-Spain1H showed a 100% offspring survival rate and a low vertical transmission rate (5%) in a pregnant mouse model [29], and no foetal death was observed in experimentally infected cattle [18]. The higher proliferation ability of Nc-Spain7 in trophoblast cells found in the present study

may be responsible for the increase in the quantity of parasites reaching the foetal tissues and, consequently, for the enhancement of parasite burdens and pathology, ultimately resulting in foetal death and abortion. These results agree with those obtained in previous studies, where higher parasite burdens in the brain and placental tissues, a wider spread and greater severity of histopathological lesions and clinical signs were observed in animals experimentally infected with Nc-Spain7 [18, 20, 50]. However, in Nc-Spain1H-infected animals, less severe lesions were observed in placentas and maternal and foetal tissues [18], which may explain the absence of abortion. In terms of dissemination in vivo, isolates with low virulence could have a lower efficiency at crossing biological barriers.

More interestingly, contrary to our observation in trophoblast cells, the behaviour of both isolates was very similar in bovine caruncular cells. Differences between isolates were limited to a slightly higher infection rate by the virulent isolate Nc-Spain7, whereas adhesion, invasion and proliferation mechanisms were very similar for both isolates. This fact has also been observed in the phylogenetically-related protozoan *T. gondii*, where comparisons between three strains showed no significant differences in their capacity to infect human placental explants [11]. The comparable behaviour showed by different virulence isolates, together with the lower invasion, infection and proliferation rates found in caruncular cells, leads us to hypothesize that isolates may have been selected because of a low virulence in the maternal part of the placenta despite their differences in virulence traits in other host cells, including other placental cells such as trophoblasts. This reduced virulence in the caruncle may facilitate, on the one hand, evasion from maternal immunity and the placental damage caused by parasite multiplication, leading to the abortion. On the other hand, this behaviour may facilitate vertical transmission to the progeny, which is the main route of transmission for *N. caninum.* In fact, Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy but congenitally infected calves, as described above.

Conclusions

This is the first study where an in vitro model of *N. caninum* infection has been implemented in bovine placental cells. Our findings confirm a differential competency of two isolates of *N. caninum* with different virulence to proliferate in bovine trophoblast cells. However, bovine caruncular cells were the first cell line assayed where different virulence isolates showed similar invasion, adhesion and proliferation kinetics. The low replication of both isolates in the maternal side of the placenta may facilitate the evasion of the inmune response by the parasite, allowing their transplacental transmission. This fact may have constituted an evolutionary advantage for these isolates. Remarkably, limited parasite invasion and growth in caruncular cells suggest a putative barrier function for this cell type in the placenta, although early parasite egress may facilitate transmission to offspring.

Furthermore, our results confirm the role of foetal trophoblasts as target cells for *N. caninum*. Future research to determine the differences in surface receptors and cell junctions between both placental cell types are needed. In addition, studies focused on co-cultures of maternal and foetal cells may be helpful as a model to study parasite transport across the maternal epithelium as part of the bovine placental barrier. Finally, the existence of differences in local immunomodulation and cellular mechanisms that take place in the placenta infected by high- and low-virulence isolates of *N. caninum* should be investigated.

Abbreviations

BCEC-1: Bovine caruncular epithelial cell line 1; BVD: Bovine viral diarrhea; CInfR: Cell infection rate; C_t: Cycle threshold value; DMEM: Dulbecco's Modified Eagle Medium; F3: Bovine placental trophoblast cell line; FCS: Foetal calf serum; hpi: Hours post-infection; MOI: Multiplicity of infection; PInvR: Parasite invasion rate; $pInvR_T$: Total parasite invasion rate; qPCR: Real-time PCR; SD: Standard deviation; T_d: Doubling time; TY_{58h}: Tachyzoite yield at 58 h post-infection

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Availability of data and materials

Not applicable.

Authors' contributions

JRC, PH, ECF, MGB and LMO conceived the study and participated in its design. LJP and MGS wrote the manuscript, with interpretation of results and discussion inputs from JRC, ECF, MGB, LMO, NH and CP. LJP and MGS performed in vitro plaque and immunofluorescence assays. JRC, PH, LJP and MGS designed and performed RT-qPCR analyses. NH and CP isolated bovine trophoblast and caruncular cell lines used in the assays. LJP and MGS carried out statistical analyses and interpreted the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author details
[1]SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. [2]Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany.

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Immune response profile of caruncular and trophoblast cell lines infected by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *Neospora caninum*

Laura Jiménez-Pelayo[1†], Marta García-Sánchez[1†], Javier Regidor-Cerrillo[1], Pilar Horcajo[1], Esther Collantes-Fernández[1], Mercedes Gómez-Bautista[1], Nina Hambruch[2], Christiane Pfarrer[2] and Luis Miguel Ortega-Mora[1*]

Abstract

Background: Bovine neosporosis, one of the main causes of reproductive failure in cattle worldwide, poses a challenge for the immune system of pregnant cows. Changes in the Th-1/Th-2 balance in the placenta during gestation have been associated with abortion. Cotyledon and caruncle cell layers form the maternal-foetal interface in the placenta and are able to recognize and induce immune responses against *Neospora caninum* among other pathogens. The objective of the present work was to elucidate the immunomodulation produced by high- (Nc-Spain7) and low-virulence (Nc-Spain1H) isolates of *N. caninum* in bovine trophoblast (F3) and caruncular cells (BCEC-1) at early and late points after infection. Variations in the mRNA expression levels of toll-like receptor-2 (TLR-2), Th1 and Th2 cytokines (IL-4, IL-10, IL-8, IL-6, IL-12p40, IL-17, IFN-γ, TGF-β1, TNF-α), and endothelial adhesion molecules (ICAM-1 and VCAM-1) were investigated by RT-qPCR, and protein variations in culture supernatants were investigated by ELISA.

Results: A similar pattern of modulation was found in both cell lines. The most upregulated cytokines in infected cells were pro-inflammatory TNF-α ($P < 0.05$–0.0001) and IL-8 ($P < 0.05$–0.001) whereas regulatory IL-6 ($P < 0.05$–0.001) and TGF-β1 ($P < 0.05$–0.001) were downregulated in both cell lines. The measurement of secreted IL-6, IL-8 and TNF-α confirmed the mRNA expression level results. Differences between isolates were found in the mRNA expression levels of TLR-2 ($P < 0.05$) in both cell lines and in the mRNA expression levels ($P < 0.05$) and protein secretion of TNF-α ($P < 0.05$), which were higher in the trophoblast cell line (F3) infected with the low-virulence isolate Nc-Spain1H.

Conclusions: *Neospora caninum* infection is shown to favor a pro-inflammatory response in placental target cells in vitro. In addition, significant immunomodulation differences were observed between high- and low-virulence isolates, which would partially explain the differences in virulence.

Keywords: *Neospora caninum*, Cattle, Immune response, Placenta, Trophoblast, Caruncle, Isolates, Virulence, Cytokines

*Correspondence: luis.ortega@ucm.es
†Laura Jiménez-Pelayo and Marta García-Sánchez contributed equally to this work
[1] SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
Full list of author information is available at the end of the article

Background

Bovine neosporosis is one of the main transmissible causes of abortion in cattle worldwide [1–3]. The etiological agent of bovine neosporosis is *Neospora caninum*, an obligate intracellular parasite closely related to the zoonotic agent *Toxoplasma gondii*. Transplacental transmission is the main route of transmission in cattle [4] and the placenta can play a key role in the pathogenesis of bovine neosporosis [5, 6]. The direct damage produced by the multiplication of the parasite in placental and foetal tissues has been proposed as one of the possible causes of abortion observed during *N. caninum* infections. Importantly, the placenta is considered to be an immune regulatory organ since it acts as a modulator of foetal and maternal immune responses. In fact, an immune-mediated pathogenesis has also been suggested as a possible cause of abortion [7]. It has been shown that the multiplication of the parasite in the placenta alters the immunological balance at the maternal-foetal interface with an increase of local pro-inflammatory IFN-γ, IL-12p40 and TNF-α cytokines which could compromise the gestation, together with an increase in IL-4 and IL-10 levels [8, 9], which avoids the immunological rejection of the foetus but favours the multiplication and vertical transmission of the parasite [5, 10]. Trophoblast and caruncular cells are able to recognize pathogens and secrete cytokines and chemokines that recruit immune cells in the damaged area [11–13]. Thus, both cell types play a fundamental role in the initiation of innate immune responses at the placental level as well as in the development of an adaptative immune response for the pregnant dam and foetus.

Previous in vivo studies have shown the influence of the isolate on the dynamics and outcome of the infection in pregnant bovine models and in the cytokine profiles induced during the infection ([9, 14–16], Jiménez-Pelayo et al. unpublished data). To date, only one recent study has utilized an in vitro model consisting of immortalized bovine trophoblasts (F3) from the foetus and caruncular cells (BCEC-1) from the dam. The aim of the study was to elucidate the interactions between tachyzoites and the host cells that resemble the maternal-foetal interface of the bovine placentome while also taking into account the influence of the isolate. Maternal cells, where both isolates showed similar phenotypic traits, presented higher resistance to the infection than trophoblast cells, where the high- (Nc-Spain7) and the low-virulence (Nc-Spain1H) isolates showed marked differences in proliferation [17].

However, the interactions between the parasite and the placental target cells from an immunological point of view have not been investigated in vitro until now. Thus, the objective of the present study was to compare the immune response profiles of the bovine placental cells in vitro after the infection with two *N. caninum* isolates of different virulence. Messenger RNA expression levels of TLR-2, pro-inflammatory cytokines IL-8, IL-12p40, IL-17, IFN-γ, TNF-α, anti-inflammatory/regulatory cytokines TGF-β1, IL-4, IL-6 and IL-10 as well as ICAM-1 and VCAM-1 endothelial adhesion molecules were determined at 4 and 24 hours post-infection (hpi) in maternal caruncular (BCEC-1) and foetal trophoblast (F3) cell cultures and protein secretion was assessed in culture supernatants by ELISA.

Results

Expression profile of TLR-2

Our results showed that *N. caninum* infection for 4 h in BCEC-1 cells resulted in a significant upregulation of TLR-2 expression in Nc-Spain1H-infected cells compared with that of negative control cells (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2 = 16.2$, $df = 2$, $P = 0.0001$) and with that of BCEC-1 cells infected with the high-virulence isolate Nc-Spain7 (Mann–Whitney U-test: $U_{(8)} = 8$, $Z = 2.591$, $P = 0.0007$). In F3 cultures, statistical significance was not found at either 4 or 24 hpi between infected groups and the control group. However, comparing both isolates, lower expression of TLR-2 was found in the F3 cultures infected with Nc-Spain7 than in the F3 cultures infected with the low-virulence isolate Nc-Spain1H at 4 hpi (Mann–Whitney U-test: $U_{(8)} = 16$, $Z = 2.287$, $P = 0.0315$) (Fig. 1a).

Pro-inflammatory and regulatory cytokine modulation

The pro-inflammatory cytokines IL-8 (Kruskal–Wallis H-test: $\chi^2 = 19.52$, $df = 2$, $P < 0.0001$ in BCEC-1 and $\chi^2 = 17.56$, $df = 2$, $P = 0.0002$ in F3) and TNF-α (Kruskal–Wallis H-test: $\chi^2 = 19.73$, $df = 2$, $P < 0.0001$ in BCEC-1 and $\chi^2 = 19.4$, $df = 2$, $P < 0.0001$ in F3) were upregulated in both cell types at 4 hpi compared to the respective control groups (Fig. 1b, c). At 24 hpi, IL-8 expression was still increased in BCEC-1 cells infected by both isolates (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2 = 16.63$, $df = 2$, $P = 0.0003$ and $\chi^2 = 10.19$, $df = 2$, $P = 0.0117$ for Nc-Spain7 and Nc-Spain1H, respectively); however, the increment of IL-8 had disappeared at 24 hpi in F3-infected cells with respect to the control group. When both isolates were compared, Nc-Spain1H induced a higher expression of TNF-α than the high-virulence isolate Nc-Spain7 at 4 hpi in F3 cells (Mann–Whitney U-test: $U_{(8)} = 0$, $Z = 2.579$, $P < 0.0001$). Protein levels of the pro-inflammatory cytokines IL-8 and TNF-α were also investigated in the supernatant of control and infected cultures at different time-points post-infection. A higher secretion of IL-8 was found for both isolates in BCEC-1 cells at 24 hpi (Kruskal–Wallis

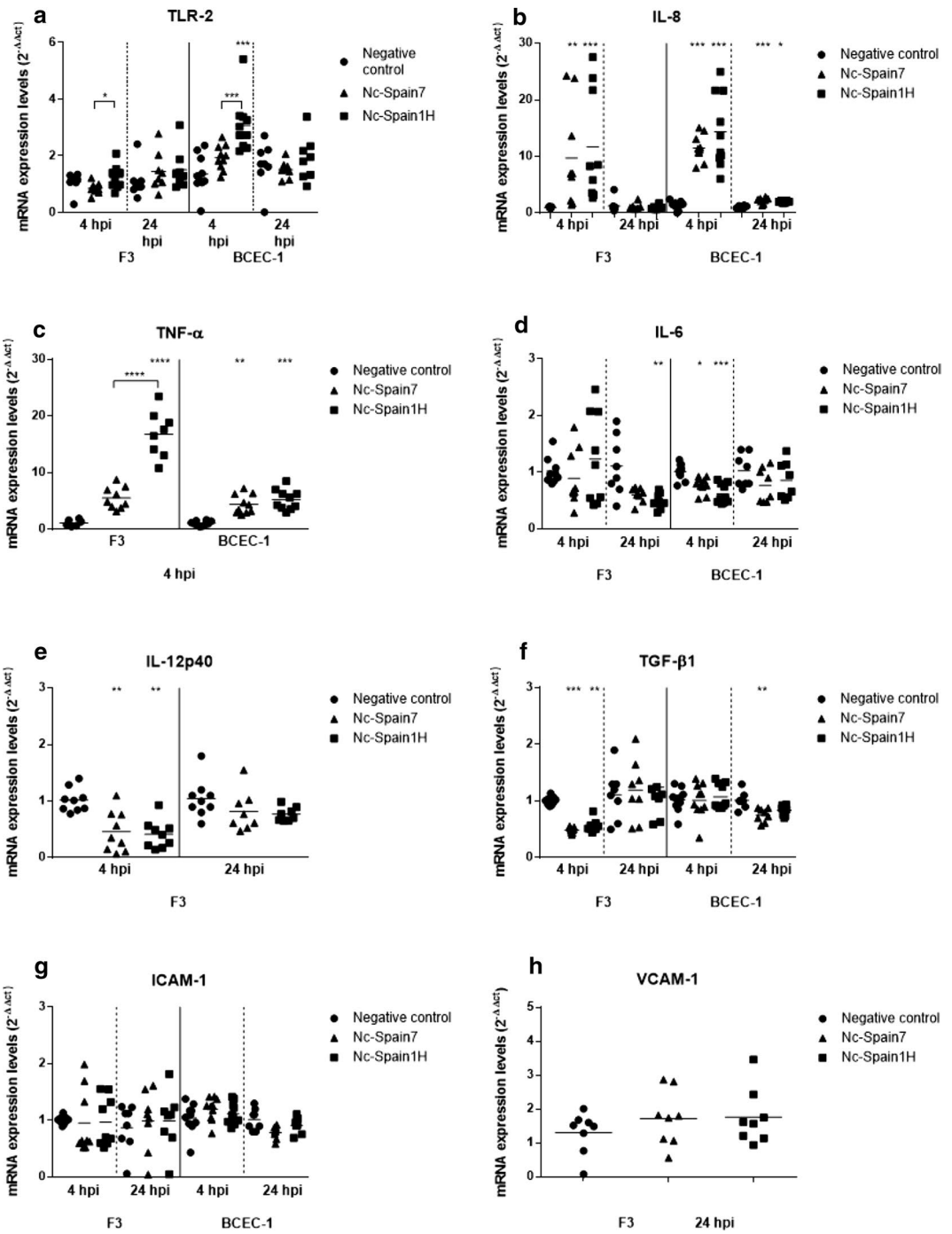

Fig. 1 TLR-2, IL-8, TNF-α, IL-6, IL-12p40, TGF-β1, ICAM-1 and VCAM-1 transcript expression. Scatter-plot graphs of relative mRNA expression levels (as x-fold change) of TLR-2 (**a**), IL-8 (**b**), TNF-α (**c**), IL-6 (**d**), IL-12p40 (**e**), TGF-β1 (**f**), ICAM-1 (**g**) and VCAM-1 (**h**) in F3 and BCEC-1 cell cultures at 4 and 24 hpi with Nc-Spain7 and Nc-Spain1H isolates. Data are represented as individual points. Horizontal lines represent median values for each group. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Unbracketed symbols represent differences with respect to the control group, while significant differences between isolates are denoted by horizontal square brackets

H-test: $\chi^2 = 15.87$, $df = 2$, $P = 0.0004$) and in F3 cells at 56 hpi (Kruskal–Wallis H-test: $\chi^2 = 13.74$, $df = 2$, $P = 0.001$) with respect to the control group (Fig. 2a, b). Secretion of TNF-α was higher in BCEC-1 cells infected by both isolates (Kruskal–Wallis H-test: $\chi^2 = 18.9$, $df = 2$, $P < 0.0001$; Fig. 2c) and in F3 cells infected by Nc-Spain1H (Kruskal–Wallis H-test followed by Dunn's multiple comparison test: $\chi^2 = 16$, $df = 2$, $P < 0.0001$) at 8 hpi, although an earlier secretion of TNF-α was also found in F3 cells infected by Nc-Spain1H ($\chi^2 = 14$, $df = 2$, $P = 0.0003$) at 4 hpi (Fig. 2d, e). As observed with the TNF-α mRNA expression, Nc-Spain1H induced a higher secretion of TNF-α than did the high-virulence isolate Nc-Spain7 at 4 hpi (Mann–Whitney U-test: $U_{(8)} = 0$, $Z = 2.736$, $P = 0.0002$) and at 8 hpi ($U_{(8)} = 0$, $Z = 2.305$, $P = 0.0002$) in the F3 cultures (Fig. 2d, e).

The expression levels of other important cytokines associated with *N. caninum* infection, such as IL-12p40 and IL-6 (Fig. 1d, e), were modified in placental cells after parasite infection. Specifically, IL-6 levels were downregulated in BCEC-1 infected by Nc-Spain1H and Nc-Spain7 at 4 hpi (Kruskal–Wallis H-test: $\chi^2 = 16.08$, $df = 2$, $P = 0.0003$) and F3 cultures infected by Nc-Spain1H at 24 hpi ($\chi^2 = 10.5$, $df = 2$, $P = 0.0052$). IL-12p40 was also

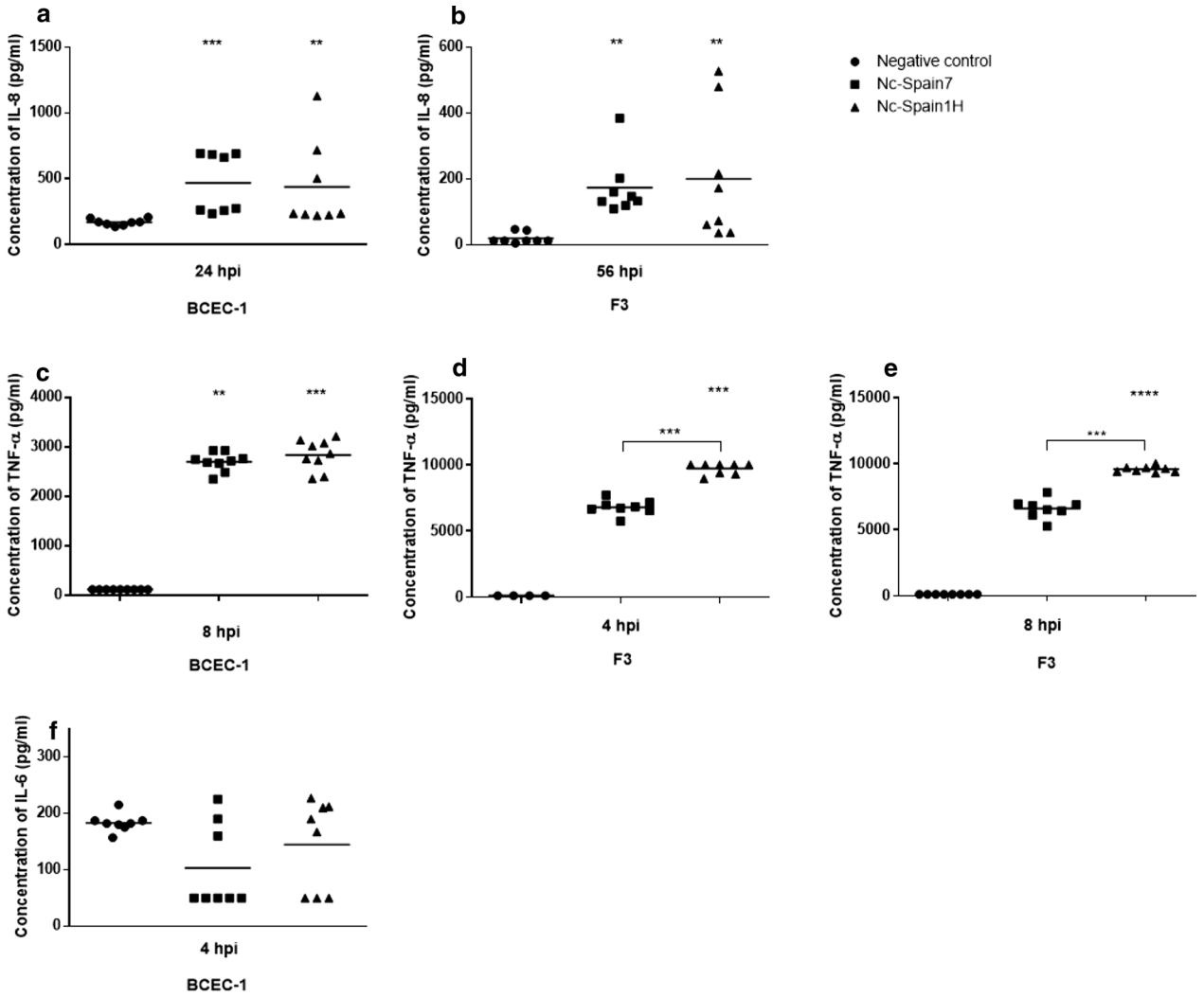

Fig. 2 IL-8, TNF-α and IL-6 secretion levels in culture supernatants. Scatter-plot graphs representing the concentration of IL-8 (pg/ml) in BCEC-1 (**a**) and F3 (**b**) supernatants infected with Nc-Spain7 and Nc-Spain1H at 24 and 56 hpi, respectively, the concentration of TNF-α (pg/ml) in the BCEC-1 supernatants at 8 hpi (**c**) and in the F3 supernatants at 4 hpi (**d**) and 8 hpi (**e**), and the concentration of IL-6 (pg/ml) in the BCEC-1 supernatants at 4 hpi (**f**). Data are represented as individual points. Horizontal lines represent median values for each group. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Unbracketed symbols represent differences with respect to the control group, while significant differences between isolates are denoted by horizontal square brackets

downregulated but only in infected F3 cultures at 4 hpi ($\chi^2 = 12.99$, $df = 2$, $P = 0.0015$). Differences between isolates in the modulation of IL-6 and IL-12p40 were not found. In addition, we observed that the caruncular cell layer did not express IL-12p40 mRNA at any time point. The decrease in the expression of IL-6 observed in infected BCEC-1 cells was confirmed by the decrease in the secretion levels of that protein found in the supernatants from BCEC-1 cultures infected with both isolates at 4 hpi, although statistically significant differences were not found (Kruskal–Wallis H-test: $\chi^2 = 2.765$, $df = 2$, $P = 0.251$), probably because of the high deviation between samples (Fig. 2f).

Finally, pro-inflammatory IL-17 and IFN-γ responses were not detected in any cell lines at 4 nor at 24 hpi.

We also studied the mRNA levels of the anti-inflammatory cytokines TGF-β1, IL-4 and IL-10. Remarkably, we observed a decrease in the expression levels of TGF-β1 in both cell lines infected with both isolates. Specifically, a decrease was observed at 4 hpi in F3 cultures (Kruskal–Wallis H-test: $\chi^2 = 18.44$, $df = 2$, $P < 0.0001$) and at 24 hpi in BCEC-1 cultures ($\chi^2 = 12.02$, $df = 2$, $P = 0.0025$) (Fig. 1f). No differences between isolates were observed in the mRNA expression levels of TGF-β1. There was not detection in bovine placental cells of the anti-inflammatory cytokine IL-4 or the regulatory cytokine IL-10 at 4 and 24 hpi.

Endothelial adhesion molecule (ICAM-1 and VCAM-1) expression

The adhesion molecule ICAM-1 was expressed by both cell lines at 4 and 24 hpi. However, only a slight decrease in the mRNA expression levels of ICAM-1 was observed in the BCEC-1 cultures infected with Nc-Spain7 at 24 hpi compared to the control group although statistical significance was not found (Kruskal–Wallis H-test: $\chi^2 = 5.894$, $df = 2$, $P = 0.0525$) (Fig. 1g). VCAM-1 expression was detected only in the F3 cultures at 24 hpi, but differences between the infected and the control groups were not found in this culture at this time point (Fig. 1h).

Results of mRNA expression levels and protein secretion from statistical tests are reported in Additional file 1: Tables S1 and S2, respectively.

Discussion

Transmission of *N. caninum* across the placenta makes this organ key in the pathogenesis of bovine neosporosis. Innate immune signalling is crucial at the maternal-foetal interface, where vertical transmission of pathogens to the foetus can have profound pathological outcomes. Trophoblasts and other cell types within the placenta may also be involved in the physiological protection of the placenta [18]. Trophoblast cells have been shown to respond to some infections by producing pro-inflammatory cytokines and chemokines and endometrial or decidual cells can produce and secrete a variety of cytokines, participating in the attraction and activation of immune effector cells [19, 20]. However, these innate immune mechanisms are unexplored at the maternal-foetal interface during *N. caninum* infection in pregnant cattle [21].

The expression of TLRs has been described in trophoblasts and other cell types within the placenta [22]. Specifically, TLR-2 was overexpressed in bovine trophoblast cell cultures at 8 hpi [23] and TLR-3, 7 and 8 have been implicated in *N. caninum* recognition in the bovine placenta [21, 24]. In our study, differential activation of TLR-2 in the F3 and BCEC-1 cultures was observed. An upregulation of TLR-2 was found in BCEC-1-infected cultures, especially in those infected with the low-virulence isolate Nc-Spain1H. The caruncular part of the placentome showed a higher expression of several TLRs, suggesting that the initial recognition of *N. caninum* at the placental level would occur in the maternal side of placenta [21]. Taking into account the data shown in Jiménez-Pelayo et al. [17], which confirm the higher proliferation of *N. caninum* in trophoblast cells, an important role of placental TLR-2 in the immune response against *N. caninum* seems plausible. TLR activation is crucial for initiating the innate immune responses responsible for the elimination of intracellular parasites such as *N. caninum*, and the signalling pathway activated by TLR-2 leads to an increase in the transcription factors NF-κβ and AP-1, which trigger the synthesis of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and IL-1β) and chemokines (IL-8, RANTES) [25].

Despite differential modulation of host TLR-2, both cell types presented a similar variation in the IL-6, TNF-α and IL-8 expression levels in infected cultures. The pro-inflammatory IL-8 and TNF-α cytokines were upregulated, and secretion of the proteins in the supernatants of both cell lines was also detected by ELISA. IL-8, a cytokine with neutrophil chemotactic/activating and T-cell chemotactic activity both in vivo and in vitro, is important in the recruitment of leukocytes to the endometrium and may be a potential mediator of placental macrophage infiltration [26], which might help to eliminate the parasite. IL-8 upregulation has already been observed in bovine umbilical vein endothelial cells (BUVECs) infected by *T. gondii* and *N. caninum* [27] as well as in bovine trophoblastic cells and placentomes from cows infected with *Brucella abortus* [28]. TNF-α is an inflammatory cytokine whose expression has also been described for epithelial cells [29]. TNF-α is expressed in all cell types of the trophoblastic lineage and provokes a variety of biological effects on placental and endometrial cell types [29]. In addition, TNF-α, through

the NF-κβ signalling pathway, coordinates the inflammatory response via the induction of other cytokines (IL-1 and IL-6) and chemokines (IL-8) and via the upregulation of adhesion molecules (ICAM-1 and VCAM-1) [30, 31], playing a role favouring protective immunity in infectious diseases [32]. There are several lines of experimental evidence indicating that TNF-α plays a role not only in immunity to *N. caninum* but also in the immunopathology of neosporosis. TNF-α expression and secretion may reduce the parasite presence in the placenta by inhibiting the intracellular multiplication of the parasite [33] and participating in parasite proliferation control mechanisms [34]; however, TNF-α expression is detrimental to pregnancy maintenance [8].

IL-6 expression levels were diminished in infected cultures of BCEC-1 and F3 at 4 and 24 hpi, respectively. The classification of IL-6 as Th1 or Th2 has been considered controversial since it can have characteristics of both depending on the dose, the cellular source and the gestational stage studied [35]. Currently, the presence of IL-6 displaces the Th1/Th2 balance towards a Th2 response [36]. However, in vivo models of *N. caninum* infection have shown IL-6 upregulation [37, 38, Jiménez-Pelayo et al. unpublished data]. This response pattern may be related to a protective action that protects the foetus and allows gestation even if the animals are born infected [10]. The decrease in IL-6 observed could be explained by the following: (i) IL-6 expression levels were affected by the high antigenic dose administered (MOI 10), resulting in downregulation [39]; (ii) the time points were not adequate for detecting the peak of IL-6 expression, and the observed decrease may be the consequence of the rapid reduction in IL-6 expression after a peak of expression [40–42]; or (iii) other cell types are implicated in the upregulation of IL-6 that was observed in vivo.

The anti-inflammatory cytokine TGF-β1 was also found to be downregulated in F3 and BCEC-1 cultures at 4 and 24 hpi, respectively. Several members of the TGF-β superfamily have been suggested to regulate trophoblast cell functions, and their dysregulation has been implicated in pregnancy-associated diseases. TGF-β1 is crucial in neutralizing the inflammatory responses induced by Th1-type cytokines [5]. This effect has already been observed in previous works where the reduction of TGF-β1 was shown to be beneficial for controlling *N. caninum* growth but detrimental for the adequate maintenance of pregnancy [38, 43].

The reduction of pro-inflammatory IL-12p40 observed in trophoblast cells, together with the lack of expression of IL-12p40 in BCEC-1 cultures, disagrees with the results of previous experimental infections [8, 9, 38, 44, 45]. Similarly, the lack of expression of pro-inflammatory IFN-γ, essential for controlling parasite infection [1, 46], and anti-inflammatory IL-4 and IL-10, related to placental protection during *N. caninum* infections [8, 9], lead us to hypothesize that the upregulation of IL-12p40, IFN-γ, IL-4 and IL-10 observed in vivo could be attributed to immune cells present in the placenta, such as dendritic cells, NK cells or macrophages. Therefore, trophoblast and/or caruncular cells would not be responsible for the direct production of these cytokines, although the assayed time points may not have been appropriate for their detection.

Finally, ICAM-1 and VCAM-1 expression were not modulated by the parasite infection. These adhesion molecules participate in the recruitment of inflammatory immune cells [47] and promote the adherence of monocytes to endothelial cells [48]. The upregulation of ICAM-1 has been observed in in vitro infections with apicomplexan parasites [27, 49, 50]. The absence of modulation observed in this work may be explained by differences in the timing of the expression of ICAM-1 and VCAM-1 [27, 49, 50] or by the lack of stimuli such as the acute-phase protein C-reactive protein (CRP), which is produced by the liver in response to IL-6 [51].

As mentioned above, the parasite isolate is a key factor in the outcome of the infection. In general, differences in the modulation between high- and low-virulence isolates were not remarkable in trophoblast or caruncular cells, with the exception of the mRNA expression levels of TLR-2 and TNF-α. TLR-2 levels were more upregulated by Nc-Spain1H infection than by Nc-Spain7 infection in both cell lines, which led us to hypothesize that the high-virulence isolate would activate less of the TLR recognition system, reducing the immune responses triggered by TLR-2. The inhibition of the TLRs implicated in the recognition of *Trypanosoma cruzi* and *T. gondii* in HPCVE increased the parasite burden and, importantly, TLR-2 inhibition prevented the secretion of IL-6 and IL-10, increasing parasite damage [52, 53]. The low-virulence isolate Nc-Spain1H activates the expression of TLR-2, starting an inflammatory response, which may be the cause of the lower proliferation of this isolate [17, 54] in addition to being one of the causes that explains the higher levels of TNF-α in Nc-Spain1H-infected cells, especially in trophoblast cells. Our results suggest that differential activation of the TLRs by the isolates of differing virulences should be subject to future research since they may be responsible for the biological differences observed both in vitro and in vivo.

The low-virulence isolate Nc-Spain1H also induced higher expression of TNF-α in F3. A higher TNF-α response may more efficiently control the proliferation of Nc-Spain1H in F3 cultures, which could explain the observations made by Jiménez-Pelayo et al. [17] where lower proliferation of Nc-Spain1H was observed in these

cells. The lower expression of TNF-α observed during the early stage of infection of trophoblasts with the high-virulence isolate Nc-Spain7 supports the hypothesis that this isolate may modify by yet unknown mechanisms the pro-inflammatory response by trophoblast cells. However, how Nc-Spain7 is able to evade the immune response and maintain lower levels of TNF-α expression in F3 remains unknown. On the other hand, these results suggest that pro-inflammatory cytokines such as TNF-α could have a minor impact in placental damage than postulated in previous works [8, 55], but other mechanisms should be implicated in placental damage in vivo and the occurrence of abortion, such as the high multiplication ability showed by the high-virulence isolate Nc-Spain7 [17].

Conclusions

The results presented in this manuscript suggest that placental cells participate in the innate immune response at the maternal-foetal interface via a rapid pro-inflammatory response characterized by the overexpression of IL-8 and TNF-α and the downregulation of TGF-β1 and IL-6. Slight differences were detected when the immunomodulatory response induced by the high and low virulent *N. caninum* isolates was compared. The higher expression of TLR-2 in the F3 and BCEC-1 cells and the TNF-α in F3 cells infected with the low-virulence isolate Nc-Spain1H may indicate a higher stimulation of the immune response by this isolate or a higher immunomodulation of Nc-Spain7, which could explain the biological differences observed in vitro and in vivo. F3 and BCEC-1 cultures seem to be a good tool for the study of the TLR activation mechanisms by *N. caninum.* Finally, we observed that cytokines such as IFN-γ, IL-4 or IL-10, which are commonly upregulated in the placenta after *N. caninum* infection, are not expressed in F3 and BCEC-1 cells; we conclude that the trophoblast and caruncular epithelial cells are not implicated in the production of these cytokines in the placenta or that other pathways/cells/molecules are needed for their production.

Methods

Parasites and cell cultures

A full description of the Nc-Spain1H and Nc-Spain7 parasites and cell cultures of bovine caruncular epithelial (BCEC-1) and bovine trophoblast cells (F3) is provided in a previous report [17]. Briefly, Nc-Spain7 and Nc-Spain1H isolates were obtained from healthy, congenitally infected calves [56, 57] and tachyzoites were maintained in a MARC-145 culture as described previously [54]. The number of culture passages of both *N. caninum* isolates was limited (passages from 9 to 11) to maintain their biological in vivo behavior [58].

The BCEC-1 and F3 cell lines were kindly donated by Dr C. Pfarrer from the University of Veterinary Medicine Hannover and maintained following the protocols described in the literature [59, 60].

Infection of the cultures, collection and preservation of the samples

BCEC-1 and F3 cells were seeded in 25 cm^2 culture flasks adjusting the number of cells in order to obtain a confluent monolayer after 24 h of culture. F3 was seeded at 10^6 cells per flask, whereas BCEC-1 was seeded at a concentration of 2×10^6 cells per flask. Tachyzoites were recovered from MARC-145 cultures when most of the parasites were still inside parasitophorous vacuoles; tachyzoites were purified using disposable PD-10 Desalting Columns (G.E. Healthcare, Amersham, UK) as previously described [54]. The parasite viability was checked by trypan blue exclusion, and the tachyzoites were counted. Multiplicity of infection (MOI) of 8 (8×10^6 tachyzoites in F3 and 16×10^6 tachyzoites in BCEC-1) and 10 (10^7 tachyzoites in F3 and 2×10^7 tachyzoites in BCEC-1) from the Nc-Spain7 and Nc-Spain1H isolates, respectively, were inoculated into confluent monolayers of F3 and BCEC-1 quickly after collection. Due to the differences observed in the infection rate between isolates [17], different MOIs of each isolate were selected with the aim of obtaining cultures infected with the same quantity of each parasite at 4 and 24 hpi. This way possible differences in the modulation of the mRNA expression levels between isolates could be attributed to differences in their biological behavior and not to the differences in the parasite burden. In addition, cultures were infected with high doses of both parasites to get a high infection of the cultures at 4 and 24 hpi so that the RNA from uninfected cells did not mask possible differences in RNA expression levels induced by the infection. The flasks were incubated at 37 °C until collection of the samples. The supernatants were collected at different time points (4, 8, 24 and 56 hpi) and stored at −80 °C for the detection of proteins by ELISA. The cultures were harvested at 4 or 24 hpi by scraping, centrifugation at 1350×*g* for 15 min at 4 °C and resuspending the pellet in 300 µl of RNAlater® (Qiagen, Hilden, Germany). The samples were stored at −80 °C prior to RNA extraction.

Two independent experiments were carried out and four replicates were obtained in each experiment.

RNA extraction, reverse transcription and quantitative real-time PCR

The mRNA expression levels of TLR-2, pro-inflammatory cytokines IL-6, IL-8, IL-12p40, IL-17, IFN-γ, TNF-α, anti-inflammatory/regulatory cytokines TGF-β1, IL-4 and IL-10 as well as ICAM-1 and VCAM-1 endothelial

adhesion molecules were determined by real-time RT-PCR in the F3 and BCEC-1 cell layers infected with the high-virulence isolate (Nc-Spain7) and the low-virulence isolate (Nc-Spain1H) of *N. caninum* at an early (4 hpi) and a late (24 hpi) time point.

RNA was extracted using a commercial Maxwell® 16 LEV simplyRNA Purification kit (Promega, Madison, WI, USA) following the manufacturer's recommendations. RNA integrity was checked by 1% agarose gel and RNA concentrations were determined using a NanoPhotometer® spectrophotometer (Implen, Munich, Germany). cDNA was obtained by reverse transcription of 2.5 µg of RNA using the master mix SuperScript® VILO™ cDNA Synthesis kit (Invitrogen, Paisley, UK), which was diluted 1:20 in molecular grade water for the qPCR assays.

The PCRs were performed using 12.5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 10 pmol of each primer (except for TLR-2 primers which were used at a concentration of 22.5 pmol) and 5 µl of diluted cDNA samples in an ABI 7300 Real Time PCR System (Applied Biosystems). The primers used for the qPCR reactions are shown in Table 1. β-Actin and GAPDH were used as housekeeping

Table 1 Sequences of primers used for cytokine real-time PCR (qPCR) and standard curve data

Target[a]	Primer	Primer sequence (5′–3′)	Product size (bp)	R^2 [b]	Slope[c]
IFN-γ (NM_174086.1)	QIFN-UP[g]	GATTCAAATTCCGGTGGATG	110	0.994	(−3.47)–(−3.30)
	QIFN-RP[g]	TTCTCTTCCGCTTTCTGAGG			
TNF-α (EU276079.1)	QTNF-UP[g]	CCAGAGGGAAGAGCAGTCC	126	0.998	(−3.39)–(−3.27)
	QTNF-RP[g]	GGAGAGTTGATGTCGGCTAC			
IL-4 (M77120.1)	QIL4-UP[g]	CTGCCCCAAAGAACACAACT	169	0.995	(−3.33)–(−3.54)
	QIL4-RP[g]	GTGCTCGTCTTGGCTTCATT			
IL-6 (X68723.1)	QIL-6-UP[d]	CTGGGTTCAATCAGGCGATT	150	0.999	(−3.22)–(−3.20)
	QIL-6-RP[d]	GGATCTGGATCAGTGTTCTGA			
IL-8 (BC103310.1)	qIL8-Fw[h]	CCACACCTTTCCACCCCAAA	177	0.995	(−3.36)–(−3.23)
	qIL8-Rw[h]	CTTGCTTCTCAGCTCTCTTC			
IL-10 (NM_174088.1)	QIL10-UP[g]	TGCTGGATGACTTTAAGGGTTACC	60	0.999	(−3.27)–(−3.42)
	QIL10-RP[g]	AAAACTGGATCATTTCCGACAAG			
IL-12p40 (NM_174356.1)	QIL12-UP[g]	AGTACACAGTGGAGTGTCAG	157	0.992	(−3.39)–(−3.35)
	QIL12-RP[g]	TTCTTGGGTGGGTCTGGTTT			
IL-17 (NM_001008412.1)	qIL17bov-up[h]	GAACTTCATCTATGTCACTGC	83	0.997	(−3.30)–(−3.18)
	qIL17bov-rev[h]	TGGACTCTGTGGGATGATGA			
TGF-β1 (NM_001009400.1)	QTGF-UP[d]	GGTGGAATACGGCAACAAAA	117	0.999	(−3.60)–(−3.53)
	QTGF-RP[d]	CGAGAGAGCAACACAGGTTC			
TLR-2 (NM_001048231.1)	QTLR2-UP[e]	ACGACGCCTTTGTGTCCTAC	192	0.993	(−3.74)–(−3.38)
	QTLR2-RP[e]	CCGAAAGCACAAAGATGGTT			
ICAM-1 (NM_174348.2)	qICAM-Fw[h]	AGACCTATGTCCTGCCATCG	219	0.994	(−3.34)–(−3.30)
	qICAM-Rw[h]	GGTGCCCTCCTCATTTTCCT			
VCAM (XM_005204079.2)	qVCAM-Fw[h]	GAACTGGAAGTCTACATCTC	128	0.998	(−3.36)–(−3.32)
	qVCAM-Rw[h]	CAGAGAATCCGTGGAGCTGG			
GAPDH (NM_001034034)	GAPDH-F[f]	ATCTCGCTCCTGGAAGATG	227	0.996	(−3.67)–(−3.58)
	GAPDH-R[f]	TCGGAGTGAACGGATTCG			
β-Actin (NM_173979.3)	BACTIN-UP[g]	ACACCGCAACCAGTTCGCCAT	216	0.994	(−3.45)–(−3.36)
	BACT216-RP[g]	GTCAGGATGCCTCTCTTGCT			

[a] NCBI accession numbers are for cDNA sequences used in primer design. Primer annealing was also checked with the *Bos taurus* genomic DNA sequences (http://www.ncbi.nlm.nih.gov/nuccore)

[b] Minimum coefficient of regression (R^2) of standard curves for each PCR target in all batches of amplification

[c] Standard curve slopes. Minimum and maximum values for slopes for each PCR target in all batches of amplification

[d] Primer first described by Arraz-Solís et al. [43]

[e] Primer first described by Menzies & Ingham [61]

[f] Primer first described by Puech et al. [62]

[g] Primer first described by Regidor-Cerrillo et al. [9]

[h] Primer described in the present work for the first time

genes, obtaining comparable Ct values for all the samples. For each target gene, a seven-point standard curve was included in each batch of amplifications based on 10-fold serial dilutions starting at 10 ng/µl of plasmid DNA. The relative quantification of the mRNA expression levels (x-fold change in expression) was carried out by the comparative $2^{-\Delta\Delta Ct}$ method [63].

Measurement of cytokines in supernatants of BCEC-1 and F3 cell cultures by ELISA

Protein concentrations of the cytokines that showed variations in the mRNA expression levels were determined in the culture supernatants at 4, 8, 24 and 56 hpi using commercial ELISA kits. The levels of IL-6, IL-8 and TNF-α cytokines were measured in the supernatants of the BCEC-1 and F3 cells by sandwich ELISAs using a Bovine IL-6 ELISA Reagent kit (ESS0029; Thermo Fisher Scientific, Waltham, MA, USA), Bovine IL-8 (CXCL8) ELISA Development kit (3114-1A-6; Mabtech AB, Stockholm, Sweden) and Bovine TNF-α ELISA kit (EBTNF; Thermo Fisher Scientific) following the manufacturers' instructions. The sensitivity limits of these assays were 78 pg/ml for IL-6, 25 pg/ml for IL-8 and 100 pg/ml for TNF-α.

Statistical analysis

TLR, cytokine and endothelial adhesion molecule mRNA expression levels, as well as differences in the protein secretion between infected and control groups, were analysed using the non-parametric Kruskal–Wallis test, followed by Dunn's multiple comparison test for all pairwise comparisons. In addition, to assess differences between both infected groups a Mann–Whitney test was performed for each molecule analysed. The statistical significance for all the analyses was established with $P < 0.05$. GraphPad Prism v.5.01 software (GraphPad Software, San Diego, CA, USA) was used to perform all statistical analyses and create all the graphical illustrations.

Additional file

Additional file 1:Table S1. Statistical test results for mRNA expression levels. **Table S2.** Statistical test results for protein secretion.

Abbreviations

BCEC-1: bovine caruncular epithelial cell line 1; F3: bovine placental trophoblast cell line; qPCR: real-time polymerase chain reaction; hpi: hours post-infection; BVD: bovine viral diarrhea; DMEM: Dulbecco's modified Eagle medium; FCS: foetal calf serum; PBS: phosphate buffered saline; TLR-2: Toll-like Receptor 2; IL: interleukin; IFN: interferon; TGF: transforming growth factor; TNF: tumoral necrosis factor; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; ELISA: enzyme-linked immunosorbent assay; BUVECs: bovine umbilical vein endothelial cells; HPCVE: human placental chorionic villi explants; MOI: multiplicity of infection.

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Availability of data and materials

Not applicable.

Authors' contributions

JRC, PH, ECF, MGB and LMO conceived the study and participated in its design. LJP and MGS wrote the manuscript, with interpretation of results and discussion input from JRC, ECF, MGB, LMO, NH and CP. LJP and MGS performed in vitro infection of the cultures, collection of the samples and ELISA assays. JRC, PH, LJP and MGS designed and performed RT-qPCR analyses. NH and CP isolated bovine trophoblast and caruncular cell lines used in the assays. LJP and MGS carried out statistical analyses and interpreted the results. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1] SALUVET, Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. [2] Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, 30173 Hannover, Germany.

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